

**RESISTANCE ARTERY FUNCTION IN DIABETES AND
HYPERGLYCAEMIA**

CAROL ANN MCINTYRE

DOCTOR OF PHILOSOPHY (PhD)

**UNIVERSITY OF EDINBURGH
2003**

ABSTRACT

Diabetes mellitus is associated with an increased risk of microvascular and macrovascular complications, such as retinopathy, nephropathy, neuropathy and atherosclerosis, which account for the increased morbidity and mortality associated with this disease. There is considerable evidence that these complications are the result of vascular dysfunction, which is closely related to poor glycaemic control. This thesis studied the hypothesis that the vascular abnormalities in diabetes are the consequence of exposure to elevated blood glucose concentrations.

To date the majority of studies of vascular function in diabetes have been performed using animal models. However, results from such studies have produced conflicting results and are difficult to relate to the human condition. Therefore, it would be advantageous if vascular function could be studied in vessels isolated from a human source. Due to the irregular and unpredictable nature of obtaining human vessels it was first necessary to develop protocols using animal vessels. Vessel structure was studied using a combination of histological and immunological techniques and this was complemented by functional studies using small vessel myography. Preliminary data demonstrated that storing vessels in a physiological salt solution at 4°C did not alter endothelial or vascular smooth muscle cell function and therefore human vessels could be stored for subsequent functional analysis in the knowledge that this does not adversely affect vascular function. Exposure of rat mesenteric resistance arteries to elevated glucose had no adverse effect on endothelium-dependent relaxation. However, a selective attenuation of endothelin-1 (ET-1)-induced contraction was demonstrated, perhaps indicating alterations in ET-1 receptors.

Relatively few studies have investigated vascular function in human subcutaneous resistance arteries, hence it was necessary to develop techniques to characterise these vessels. This investigation demonstrated the existence of three distinct vessel types, large and small resistance arteries and veins, which can be distinguished on the basis of physical appearance, differences in response to transmural stretch and functional reactivity. Furthermore, endothelium-dependent relaxation in human subcutaneous resistance arteries was shown to be predominately mediated by an endothelium-derived hyperpolarising factor (EDHF), with little or no contribution from nitric oxide (NO) or prostacyclin (PGI₂). Functional investigations in resistance arteries isolated from Type 1 and Type 2 diabetic patients demonstrated unaltered endothelium-dependent and endothelium-independent relaxation. However, there was a selective alteration in contractile function which was related to the type of diabetes.

In conclusion, this study has shown that although diabetes is associated with changes in contractile function there was no evidence of impaired endothelial-dependent relaxation. The changes seen in vessels from diabetic patients did not mimic those produced by acute exposure to elevated concentrations of glucose. This suggests that if the vascular alterations observed in diabetes are due to exposure to high glucose concentrations *in vivo*, prolonged exposure (or factors not present *in vitro*) are required for abnormalities to develop.

DECLARATION

I hereby declare that the work presented in this thesis is my own and has not been submitted previously for any degree. This work was undertaken in the Department of Medicine (The Royal Infirmary Edinburgh) and the Department of Medical Sciences (Western General Hospital) of the University of Edinburgh.

Carol Ann McIntyre

(Carol Ann McIntyre)

CONTENTS

	<u>Pages</u>
Abstract	ii
Declaration	iv
Contents	v
Figures and Tables	x
Abbreviations	xvii
Acknowledgements	xx
Publications	xxi

CHAPTER 1

INTRODUCTION

1.1 STRUCTURE OF THE VESSEL WALL	2
1.1.1. ADVENTITIA.....	4
1.1.2. MEDIA	4
1.1.3. INTIMA	4
1.2 THE INFLUENCE OF THE ENDOTHELIUM ON VASCULAR TONE.....	5
1.2.1. ENDOTHELIUM-DERIVED RELAXING FACTORS.....	5
1.2.1.1. Nitric Oxide.....	5
1.2.1.2. Prostacyclin	8
1.2.1.3. Endothelium-derived hyperpolarising factor	9
1.2.2. ENDOTHELIUM-DERIVED VASOCONSTRICTORS	9
1.2.2.1. Endothelin	10
1.3. REGULATION OF VASCULAR TONE.....	11
1.3.1. SMOOTH MUSCLE CELL CONTRACTION	11
1.3.2. SMOOTH MUSCLE CELL RELAXATION.....	14
1.4. DIABETES MELLITUS	17
1.4.1. TYPE 1 DIABETES MELLITUS.....	17
1.4.2. TYPE 2 DIABETES MELLITUS	18
1.4.3. THE PATHOPHYSIOLOGY OF DIABETES MELLITUS.....	19
1.4.4. COMPLICATIONS OF DIABETES MELLITUS	20
1.4.4.1. Macrovascular disease	21
1.4.4.2. Microvascular disease	21
1.4.4.2.1. Diabetic Retinopathy.	21
1.4.4.2.2. Diabetic Nephropathy.	22
1.4.4.2.2. Diabetic Neuropathy.	23
1.5. EVIDENCE OF VASCULAR DYSFUNCTION IN DIABETES	

MELLITUS	23
1.5.1. ENDOTHELIUM-DEPENDENT VASCULAR RESPONSES IN DIABETES	25
1.5.2. HUMAN STUDIES	25
1.5.2.1 <i>In vivo studies.</i>	25
1.5.2.2 <i>In vitro studies.</i>	30
1.5.3. ANIMAL STUDIES	32
1.6. DEVELOPMENT OF DIABETIC MICROVASCULAR COMPLICATIONS; THE ROLE OF HYPERGLYCAEMIA.....	34
1.6.1. ALDOSE REDUCTASE AND THE POLYOL PATHWAY.	34
1.6.2. VASOCONSTRICTOR PROSTANOIDS.	35
1.6.3. FREE RADICALS.....	36
1.6.4. PROTEIN KINASE C.	37
1.6.5. ADVANCED GLYCOSYLATION END PRODUCTS.....	37
1.7. HYPOTHESIS AND AIMS.	39

CHAPTER 2

METHODS

2.1 SMALL VESSEL MYOGRAPHY.....	41
2.1.1. INTRODUCTION.	41
2.1.2. ISOLATION OF SMALL RESISTANCE ARTERIES	43
2.1.2.1. <i>Rat mesenteric vessels.</i>	43
2.1.2.2. <i>Human vessels: tissues from gluteal fat biopsies.</i>	44
2.1.3. MOUNTING AND NORMALISATION OF VESSELS IN THE MYOGRAPH.....	44
2.1.3.1. <i>Mounting of the vessel in the myograph.</i>	44
2.1.3.2. <i>Determination of normalised lumen using small vessel myograph.</i>	47
2.1.4. ASSESSMENT OF VESSEL VIABILITY	48
2.1.5. DE-ENDOTHELIALISATION.....	49
2.1.5.1. <i>Removal of endothelium using a human hair.</i>	50
2.1.6. FUNCTIONAL ANALYSIS.....	51
2.1.6.1. <i>Vasoconstriction</i>	51
2.1.6.2. <i>Vasodilatation</i>	51
2.1.7. STATISTICS	52
2.1.8. DRUGS.....	52
2.2. MORPHOLOGICAL ANALYSIS.....	54
2.2.1. FIXATION OF VESSELS.	54
2.2.2. HAEMATOXYLIN AND EOSIN	55
2.2.3. IMMUNOHISTOCHEMISTRY.....	56
2.2.3.1. <i>Immunohistochemical Protocol</i>	56
2.2.4. SOLUTIONS.	60
2.2.4.1. <i>Haematoxylin and eosin</i>	60
2.2.4.2. <i>Immunohistochemistry.</i>	60

CHAPTER 3

EFFECT OF PROLONGED COLD STORAGE ON RAT MESENTERIC ARTERIAL REACTIVITY

3.1. INTRODUCTION	63
--------------------------------	-----------

3.2. AIM	64
3.3. METHODS.....	64
3.3.1. EXPERIMENTAL SET-UP	64
3.3.2. EXPERIMENTAL PROTOCOL.....	65
3.3.3. DRUGS	66
3.3.4. STATISTICS	66
3.4. RESULTS	67
3.4.1. VASOCONSTRICTOR RESPONSES.....	67
3.4.2. RESPONSES TO VASODILATOR AGONISTS.	70
3.5. DISCUSSION.....	70
3.5.1. VASODILATOR FUNCTION.	74
3.5.2. VASOCONSTRICTOR FUNCTION.....	78

CHAPTER 4

THE EFFECT OF ELEVATED GLUCOSE AND INSULIN ON FUNCTION OF RAT MESENTERIC RESISTANCE ARTERY

4.1. INTRODUCTION	83
4.2. AIM	84
4.3. METHODS.....	85
4.3.1. MEASUREMENT OF VASCULAR REACTIVITY	85
4.3.2. EXPERIMENTAL PROTOCOL.....	85
4.3.3. DRUGS	87
4.3.4. STATISTICS	87
4.4. RESULTS	88
4.4.1. EFFECT OF ELEVATED CONCENTRATIONS OF GLUCOSE OR MANNITOL.....	88
4.4.2. EFFECT OF INCUBATION TIME.....	91
4.4.3. EFFECT OF ELEVATED CONCENTRATIONS OF INSULIN	94
4.5. DISCUSSION.....	94
4.5.1. EFFECT OF ELEVATED GLUCOSE CONCENTRATIONS	94
4.5.2. EFFECT OF INCUBATION TIME	100
4.5.3. EFFECT OF ELEVATED CONCENTRATIONS OF INSULIN	101

CHAPTER 5

FUNCTIONAL AND MORPHOLOGICAL CHARACTERISTICS OF VESSELS ISOLATED FROM HUMAN SUBCUTANEOUS FAT BIOPSIES.

5.1. INTRODUCTION	105
5.2. AIMS.....	107

5.3. METHODS.....	107
5.3.1. SUBJECTS	107
5.3.2. MORPHOLOGICAL INVESTIGATIONS	108
5.3.3. FUNCTIONAL INVESTIGATIONS.....	109
5.3.3.1. <i>Functional Characteristics of Small and Large Resistance Arteries and Veins.....</i>	<i>109</i>
5.3.3.2. <i>Functional Identification of Mediators of Endothelium-Dependent Relaxation in Small Resistance Arteries.....</i>	<i>110</i>
5.3.4. DRUGS	111
5.3.5. STATISTICS	111
5.4. RESULTS	112
5.4.1. MORPHOLOGY	112
5.4.2. NORMALISATION.....	114
5.4.3. VASCULAR FUNCTION.....	115
5.4.3.1. <i>Functional Characteristics of Large and Small Resistance Arteries and Veins.....</i>	<i>115</i>
5.4.3.2. <i>Functional Identification of Mediators of Endothelium-Dependent Relaxation of Human Small Resistance Arteries</i>	<i>120</i>
5.4.3.2.1. Effect of cyclooxygenase inhibition.....	120
5.4.3.2.2. Effect of NOS inhibition.....	123
5.4.3.2.3. Effect of potassium channel blockade.....	123
5.5. DISCUSSION.....	125
5.5.1. VESSEL IDENTIFICATION	125
5.5.2. ROLE OF THE L-ARGININE/NO PATHWAY IN ACH RELAXATION.....	127
5.5.3. ROLE FOR PROSTANOIDS IN ACH RELAXATION	128
5.5.4. ROLE FOR EDHF IN ACH RELAXATION.....	129

CHAPTER 6

EFFECT OF DIABETES MELLITUS ON HUMAN MICROVASCULAR REACTIVITY *IN VITRO*

6.1. INTRODUCTION	135
6.2. AIMS.....	137
6.3. METHODS.....	137
6.3.1. SUBJECTS	137
6.3.1.1. <i>Type 1 diabetes</i>	<i>137</i>
6.3.1.2. <i>Type 2 diabetes</i>	<i>138</i>
6.3.1.3. <i>Patient Assessment.....</i>	<i>138</i>
6.3.2. ANALYSIS OF RESISTANCE ARTERY FUNCTION	138
6.3.3. PROTOCOL	139
6.3.4. STATISTICS	140
6.3.5. DRUGS	141
6.4. RESULTS	141
6.4.1. TYPE 1 DIABETES.....	141
6.4.1.1. <i>Responses to Vasodilators.....</i>	<i>143</i>
6.4.1.2. <i>Responses to Vasoconstrictors</i>	<i>143</i>
6.4.2. INFLUENCE OF ELEVATED GLUCOSE CONCENTRATIONS.....	148
6.4.2.1. <i>Responses to Vasodilators.....</i>	<i>148</i>
6.4.2.2. <i>Responses to Vasoconstrictors</i>	<i>148</i>

6.4.3.	TYPE 2 DIABETES.....	157
6.4.3.1.	<i>Responses to Vasodilators.....</i>	157
6.4.3.2.	<i>Responses to Vasoconstrictors.....</i>	157
6.5.	DISCUSSION.....	163
6.5.1.	TYPE 2 DIABETES.....	163
6.5.2.	TYPE 1 DIABETES.....	164
6.5.2.1.	<i>Influence of elevated glucose concentrations.....</i>	168
6.5.3.	CONCLUSIONS.....	170

CHAPTER 7

GENERAL DISCUSSION

172

REFERENCES

178

Published Articles

FIGURES AND TABLES

CHAPTER ONE

Figures

Figure 1.1. Schematic longitudinal section of a small artery showing the three layers of the vascular wall.

Figure 1.2. Generation of nitric oxide (NO) in the vascular endothelium.

Figure 1.3. Mechanisms of contraction in vascular smooth muscle cells.

Figure 1.4. Mechanisms of agonist-induced relaxation in vascular smooth muscle cells.

Tables

Table 1.1. Investigation of human endothelial function in Type 1 diabetic subjects, studied by venous occlusion plethysmography (VOP), vascular ultrasound (US) and isolated vessel techniques, mainly small vessel myography (SVM).

Table 1.2. Investigation of human endothelial function in Type 2 diabetic subjects, studied by venous occlusion plethysmography (VOP), vascular ultrasound (US) and isolated vessel techniques, mainly small vessel myography (SVM).

CHAPTER TWO

Figures

Figure 2.1. Schematic diagram of the wire myograph, where vessel segments are threaded on two 40µm stainless steel wires that are fastened to a micrometer and a force transducer, for measurement of isometric tension. (Figure taken from Mulvany & Aalkjaer, 1990).

Figure 2.2. Schematic diagram showing the three-step indirect avidin biotinylated enzyme complex (ABC) immunohistochemical technique. The primary antibody (1° Ab), directed against the antigen (Ag), is incubated overnight at 4°C. A secondary biotinylated antibody (2° Ab) is added, followed by addition of the preformed ABC conjugated to peroxidase (Δ). To visualise the sites of positive binding, the chromagen 3,3-diaminobenzidine tetrahydrochloride (DAB), which is the substrate for peroxidase, is added, producing an insoluble brown precipitate.

Tables

Table 2.1 A list of the drugs used throughout the course of this thesis.

Table 2.2 A list of the antibodies used for immunohistochemical staining of the endothelium, vascular smooth muscle and localisation of ET-1.

CHAPTER THREE

Figures

Figure 3.1. The effect of cold storage on the cumulative concentration response curves to (a) acetylcholine (ACh; 10^{-9} - 3×10^{-5} M) and (b) 3-morpholinosydnonimine (SIN-1; 10^{-9} - 10^{-4} M) in rat mesenteric resistance arteries. Each point represents mean \pm s.e.mean, n=10. ■ Day 0, ○ Day 4.

Tables

Table 3.1. The effect of prolonged cold storage on the viability of rat mesenteric resistance arteries. Values are mean \pm s.e.mean, n numbers in parentheses.

Table 3.2. The effects of cold storage on the (a) maximum contraction (mN/mm) and (b) sensitivity (pD_2) of rat 3rd order mesenteric arteries to vasoconstrictors. Results are shown as mean \pm s.e.mean, n=10.

Table 3.3. The effect of prolonged cold storage on the magnitude of noradrenaline (3 μ M)-induced precontraction obtained in the construction of CCRC's to the vasodilators ACh and SIN-1 in rat mesenteric resistance artery.

Table 3.4. The effect of prolonged cold storage on (a) maximum relaxation (%) and (b) sensitivity ($-\log IC_{50}$) of rat 3rd order mesenteric arteries to vasodilators.

CHAPTER FOUR

Figures

Figure 4.1. Concentration-response curves to (a) endothelin-1, (b) noradrenaline and (c) potassium in rat mesenteric resistance arteries following 1hr incubation in normal PSS (5.5mM (▲), n=11-13) or PSS containing elevated concentrations of glucose (15mM (□), n=10; 20mM (○), n=9-10; 44mM (△), n=10) or mannitol (20mM (◇), n=6-9). Each point represents mean \pm s.e.mean.

Figure 4.2. Concentration-response curves to (a) acetylcholine, (b) A23187 and (c) 3'-morpholinosydnonimine (SIN-1) in rat mesenteric resistance arteries following 1hr incubation in normal PSS (5.5mM (▲), n=11-13) or PSS containing elevated concentrations of glucose (15mM (□), n=10; 20mM (○), n=6-10; 44mM (△), n=8-10) or mannitol (20mM (◇), n=5-9). Each point represents mean ± s.e.mean and is expressed as % relaxation of 3μM NA-induced tone.

Figure 4.3. Concentration-response curves to the vasoconstrictors (a) endothelin-1, (b) noradrenaline and (c) K⁺ in rat mesenteric resistance arteries following 1hr incubation in normal PSS (σ, n=10-12) or PSS containing an elevated concentration (1000mU/l) of insulin (□, n=10-12) Each point represents mean ± s.e.mean.

Figure 4.4. Concentration-response curves to the vasodilators (a) acetylcholine, (b) A23187 and (c) 3'-morpholinosydnonimine (SIN-1) in rat mesenteric resistance arteries following 1hr incubation in normal PSS (σ, n=10-12) or PSS containing 1000mU/l of insulin (□, n=10-12) Each point represents mean ± s.e.mean.

Tables

Table 4.1. Maximum contraction (E_{max}, mN/mm) and sensitivity (pD₂) to vasoconstrictor agonists in rat mesenteric resistance arteries following (a) 1 hr or (b) 4 hr incubation in normal PSS or PSS containing elevated concentrations of glucose or mannitol.

Table 4.2. Maximum relaxation (E_{max}, % reversal of 3μM NA-induced precontraction) and sensitivity (-logIC₅₀) to vasodilator agonists in rat mesenteric resistance arteries following (a) 1hr or (b) 4hr incubation in normal PSS or PSS containing elevated concentrations of glucose or mannitol.

Table 4.3. Maximum contraction (E_{max}, mN/mm) and sensitivity (pD₂) to vasoconstrictor agonists in rat mesenteric resistance arteries following incubation in normal PSS or PSS containing an elevated concentration of insulin (1000mU/l).

Table 4.4. Maximum relaxation (E_{max}, % reversal of NA-induced precontraction) and sensitivity (-logIC₅₀) to vasodilator agonists in rat

mesenteric resistance arteries following 1hr incubation in normal PSS or PSS containing an elevated concentration of insulin (1000mU/l).

CHAPTER FIVE

Figures

Figure 5.1. Physical and morphological differences between small resistance arteries (A), veins (B) and large resistance arteries (C) isolated from human subcutaneous fat biopsies. (i) light microscopy showing differences in physical characteristics between the three vessel types. (ii) haematoxylin and eosin staining showing media (M), lumen (L) and adventitia (A). (iii) and (iv) immunohistochemical localisation of smooth muscle (SM) and endothelium (E) using monoclonal antibodies against α -smooth muscle actin and ulex europaeus agglutinin, respectively. Magnification x200.

Figure 5.2. Concentration-response curves to (a) noradrenaline, (b) endothelin-1 and (c) K^+ in small resistance arteries (●, n=7), veins (▲, n=5) and large resistance arteries (■, n=7) isolated from human subcutaneous fat biopsies. Each point represents mean \pm s.e.mean.

Figure 5.3. Concentration-response curves to the vasodilators (a) acetylcholine, (b) bradykinin, (c) A23187 and (d) 3'-morpholinomethylsydnorimine (SIN-1) in small resistance arteries (●, n=7), veins (▲, n=5) and large resistance arteries (■, n=7) isolated from human subcutaneous fat biopsies. Each point represents mean \pm s.e.mean.

Figure 5.4. Trace demonstrating the effect of acetylcholine (ACh) in a human small resistance artery submaximally precontracted with noradrenaline (NA) before (a) and after (b) removal of the endothelium. W-washout.

Figure 5.5. Concentration-response curves to acetylcholine in isolated human resistance arteries before (■) and after (○) incubation with either (a) indomethacin (10 μ M for 45min), (b) L-NOARG (100 μ M for 45min) or (c) L-NOARG plus ChTx (50nM for 10min) and apamin (30nM for 10min). Each point represents mean \pm s.e.mean, n=6 for each group.

Tables

Table 5.1. Maximum contraction (E_{max}) of small and large resistance arteries and veins to noradrenaline (NA), endothelin-1 (ET-1) and KPSS.

Table 5.2. Magnitude of precontraction to NA ($3\mu\text{M}$), in human small resistance arteries, before and after incubation with either indomethacin ($10\mu\text{M}$ for 45min), L-NOARG ($100\mu\text{M}$ for 45min) or L-NOARG plus ChTx (50nM for 10min) and apamin (30nM for 10min).

CHAPTER SIX

Figures

Figure 6.1. Cumulative concentration-response curves to the vasodilator agonists (a) acetylcholine (ACh; $n=12$), (b) bradykinin (BK; $n=8$), (c) A23187 ($n=8-10$) and (d) SIN-1 ($n=11-12$) in human resistance arteries isolated from patients with type 1 diabetes (\circ) and from non-diabetic controls (\blacksquare). Results as expressed as % relaxation of vessels initially precontracted with $3\mu\text{M}$ NA and shown as mean \pm s.e.mean.

Figure 6.2. Cumulative concentration-response curves to vasoconstrictor agonists (a) endothelin-1 (ET-1), (b) noradrenaline (NA) and (c) potassium (K^+) in human resistance arteries isolated from patients with type 1 diabetes (\square) and non-diabetic controls (\blacksquare). Each point represents mean \pm s.e.mean, $n=12$.

Figure 6.3. Cumulative concentration-response curves to the vasodilator agonists (a) acetylcholine (ACh), (b) bradykinin (BK) and (c) SIN-1, in resistance arteries isolated from healthy volunteers and incubated in the presence of 5.5mM (Control, \blacksquare $n=6$) or 20mM (\square , $n=6$) glucose. Results are expressed as % relaxation of vessels initially precontracted with $3\mu\text{M}$ NA and shown as mean \pm s.e.mean.

Figure 6.4. Cumulative concentration-response curves to the vasodilator agonists (a) acetylcholine (ACh), (b) bradykinin (BK) and (c) SIN-1 in resistance arteries isolated from patients with type 1 diabetes incubated in the presence of 5.5mM (\bullet ; control) and 20mM (\circ ; elevated) glucose. Results are expressed as % relaxation of vessels initially precontracted with $3\mu\text{M}$ NA and shown as mean \pm s.e.mean, $n=8$.

Figure 6.5. Cumulative concentration-response curves to vasoconstrictor agonists (a) noradrenaline (b) endothelin-1 and (c) high potassium, in resistance arteries isolated from healthy volunteers and incubated in the presence of 5.5 mM (Control, ■ n=6) or 20 mM (□, n=6) glucose for 1 hr. Results are expressed as force per unit length of tissues (mN/mm) and shown as mean \pm s.e.mean.

Figure 6.6. Cumulative concentration-response curves to vasoconstrictor agonists (a) noradrenaline (b) endothelin-1 and (c) high potassium, in resistance arteries isolated from patients with type 1 diabetes and incubated in the presence of 5.5 mM (Control, ○ n=8) or 20 mM (●, n=8) glucose. Results are expressed as force per unit length of tissues (mN/mm) and shown as mean \pm s.e.mean.

Figure 6.7. Cumulative concentration-response curves to the vasodilator agonists (a) acetylcholine, (b) bradykinin, (c) A23187 and (d) SIN-1 in human resistance arteries isolated from patients with type 2 diabetes (○, n=8) and non-diabetic controls (■, n=10). Results as expressed as % relaxation of vessels initially precontracted with 3 μ M NA and shown as mean \pm s.e.mean.

Figure 6.8. Cumulative concentration-response curves to vasoconstrictor agonists (a) noradrenaline (b) endothelin-1 and (c) high potassium, in resistance arteries isolated from patients with type 2 diabetes (○, n=8) and non-diabetic controls (■, n=10). Results are expressed as force per unit length of tissues (mN/mm) and shown as mean \pm s.e.mean.

Tables

Table 6.1. Baseline characteristics of control subjects and patients with type 1 diabetes.

Table 6.2. Maximum relaxation (% reversal of 3 μ M NA-induced tone) and sensitivity (-LogIC₅₀ values) from concentration-response curves obtained to the vasodilator agonists in arteries from patients with type 1 diabetes and controls.

Table 6.3. Maximum contraction (a) and sensitivity (b) values from concentration-response curves obtained to the vasoconstrictor agonists in arteries isolated from patients with type 1 diabetes and non-diabetic controls.

Table 6.4. Characteristics of non-diabetic control subjects (n=6) and patients with type 1 diabetes (n=8) who provided vessels for incubation with control (5.5mM) and elevated (20mM) glucose.

Table 6.5. Maximum relaxation (% reversal of 3 μ M NA-induced tone) and sensitivity (-LogIC₅₀ values) from concentration-response curves obtained to the vasodilator agonists in arteries from (a) non-diabetic control subjects (n=6) and (b) patients with type 1 diabetes (n=8) after incubation with either normal (5mM) or elevated (20mM) glucose.

Table 6.6. Maximum contraction (a) and sensitivity (b) values from concentration-response curves obtained to the vasoconstrictor agonists in arteries isolated from non-diabetic controls incubated with either normal (5mM) or high (20mM) glucose for 1 hour.

Table 6.7. Maximum contraction (a) and sensitivity (b) values from concentration-response curves obtained to the vasoconstrictor agonists in arteries isolated from patients with type 1 diabetes incubated with either normal (5mM) or high (20mM) glucose for 1 hour.

Table 6.8. Baseline characteristics of 8 male patients with type 2 diabetes and 10 age and sex-matched non-diabetic control subjects.

Table 6.9. Maximum relaxation (% reversal of 3 μ M NA-induced tone) and sensitivity (-LogIC₅₀ values) from concentration-response curves obtained to the vasodilator agonists in the arteries from patients with type 2 diabetes and controls.

Table 6.10. Maximum contraction (a) and sensitivity (b) to vasoconstrictor agonists in isolated resistance arteries from patients with type 2 diabetes (n=8) and non-diabetic control subjects (n=10).

ABBREVIATIONS

A23187	Calcium ionophore A23187 (calcimycin)
ACh	Acetylcholine
α -SMA	Alpha-smooth muscle actin
Ang	Angiotensin II
ATP	Adenosine triphosphate
BK	Bradykinin
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CaCl_2	Calcium Chloride
$[\text{Ca}^{2+}]_i$	Intracellular calcium ion concentration
cAMP	3'5'-cyclic adenosine monophosphate
CCRC	Cumulative Concentration Response Curve
cGMP	Cyclic guanosine monophosphate
CO_2	Carbon dioxide
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAG	Diacylglycerol
E	Endothelium
ECE	Endothelin converting enzyme
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
EEL	External elastic lamina
E _{max}	Maximum Response
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ET _A	Endothelin A receptor
ET _B	Endothelin B receptor
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
IEL	Internal elastic lamina

IDDM	Insulin-dependent diabetes mellitus
iNOS	Inducible nitric oxide synthase
IP ₃	Inositol 1,4,5-trisphosphate
KH ₂ PO ₄	Potassium dihydrogen orthophosphate
L	Lumen
L-NAME	L-N ^G -nitroarginine methyl ester
L-NMMA	L-N ^G monomethyl arginine
L-NOARG	N ^G -nitro-L-arginine
LRA	Large resistance artery
MLCK	Myosin light chain kinase
MgSO ₄	Magnesium sulphate
μm	Micrometer
(n)	Number of subjects
Na ⁺ /K ⁺ ATPase	Sodium-potassium adenosine triphosphatase
NaCl	Sodium Chloride
NaHCO ₃	Sodium hydrogen carbonate
NEFA	Non-esterified fatty acid
NIDDM	Non-insulin-dependent diabetes mellitus
NO	Nitric oxide
NOS	Nitric oxide synthase
NOSII	Inducible nitric oxide synthase
NOSIII	Endothelial nitric oxide synthase
PBS	Phosphate buffered saline
PG	Prostaglandin
PKC	Protein kinase C
PLC	Phospholipase C
SEM	Standard error of the mean
SGC	Soluble guanylate cyclase
SIN-1	3'-Morpholinomethylsydnorimine
SNP	Sodium nitroprusside
SMC	Smooth muscle cell
SRA	Small resistance artery

SVM	Small vessel myography
TBS	Tris buffered saline
US	Ultrasound
V	Vein
VOP	Venous occlusion plethysmography
WHO	World Health Organisation

ACKNOWLEDGEMENTS

I would like to thank my principal supervisor, Dr. P.W.F. Hadoke, for his support and guidance over the last four years and all the assistance he has provided me with in the writing of my thesis. I would also like to thank my assistant supervisors, Professor B. R. Walker and Professor J. R. Seckl, for their contributions and ensuring that I submitted my thesis. I am indebted to all three for their assistance both during and in the writing up of my thesis.

My thanks also extends to Dr. B.C. Williams for his continued involvement in the project and to Dr. J. A. McKnight and Mr A. Elliott, for their involvement in recruiting human subjects for my studies. This work formed the backbone of my thesis and without their help the study would have been unable to continue.

I would also like to thank Dr. G. A. Gray and her team for the use of their lab and their assistance in developing the immunological and histological techniques used throughout the course of this study.

On a more personal level, I would also like to thank my parents and my husband Mark, for putting up with me over the last four years. Huge thanks go to them for their continuing support, both morally and financially, and encouragement.

PUBLICATIONS & PRESENTATIONS

Papers

- McIntyre, C.A., Williams, B.C., McKnight, J., Lindsay, M. and Hadoke, P.W.F. (1998) Preservation of vascular function in rat mesenteric resistance arteries following cold storage, studied by small vessel myography. *Br. J. Pharmacol.*, **123**, 1555-1560.
- McIntyre, C.A., Hadoke, P.W.F., Williams, B.C., Lindsay, R.M., Elliott, A.I. and McKnight, J.A. (2001) Selective enhancement of sensitivity to endothelin-1 despite normal endothelium-dependent relaxation in subcutaneous resistance arteries isolated from patients with Type 1 diabetes. *Clinical Science*, **100** (3), 311-318.
- McIntyre, C.A., Buckley, C.H., Andrews, R.C., Sandeep, T.C., Jones, G.C., Elliot, A., Gray, G.A., Williams, B.C., McKnight, J., Walker, B.R. and Hadoke, P.W.F. (2001) Endothelium-derived hyperpolarising factor and potassium use different mechanisms to induce relaxation of human sub-cutaneous resistance arteries. *Br. J. Pharmacol.*, **133**, 902-908.
- Hadoke, P.W.F., McIntyre, C.A., Gray, G.A., and Buckley C.H. (2001) Functional heterogeneity of large and small resistance arteries isolated from biopsies of sub-cutaneous fat: implications for investigation of vascular pathophysiology. *Gen. Pharmacol.*, **35** (3), 119-127.

Published Abstracts

- McIntyre, C.A., Williams, B.C., McKnight, J., Lindsay, M. and Hadoke, P.W.F. (1996) Conservation of endothelial cell response in rat mesenteric artery stored in physiological salt solution (PSS) at 4°C. *Br. J. Pharmacol.* **119**, Proc. Suppl., 318P.
- McIntyre, C.A., Williams, B.C., Hadoke, P.W.F., Lindsay, M. and McKnight, J. (1997) Acute effects of elevated glucose *in vitro* on rat mesenteric arterial reactivity. *Diabetic Medicine*, **14**, Suppl. 1, S62, P166a.
- McIntyre, C.A., Hadoke, P.W.F., Williams, B.C., Lindsay, R.M., Elliot, A. and McKnight, J.A., (1998). Endothelial and vascular smooth muscle cell function in resistance arteries isolated from patients with insulin-dependent diabetes mellitus. *Diabetic Medicine*, **15**, Suppl. 2, S16, P7.

- McIntyre, C.A., Williams, B.C., Andrews, R.C., McKnight, J.A., Walker, B.R. and Hadoke, P.W.F. (1998). Impaired receptor-independent vasoconstriction in resistance arteries isolated from patients with non insulin-dependent diabetes mellitus. *Diabetic Medicine*, **15**, Suppl. 2, S16, P6.
- McIntyre, C.A., Andrews, R.C., Elliot, A., Gray, G.A., Williams, B.C., McKnight, J., Walker, B.R. and Hadoke, P.W.F. (1998) Endothelium-derived hyperpolarising factor mediates to a large extent acetylcholine-induced relaxation of human subcutaneous resistance arteries. *Br. J. Pharmacol.*, **125**, Proc. Suppl., 16P.
- McIntyre_C.A., Williams_B.C., Elliot_A., Andrews_R.C., Walker_B.R., McKnight_J.A., and Hadoke_P.W.F. (1999) Maintained endothelial and vascular smooth muscle cell function in subcutaneous resistance arteries isolated from diabetic patients. *Diabetes*, **48**, No.S1, 1457
- Hadoke, P.W.F., McIntyre, C.A., Jones, G.C., Williams, B.C., Elliot, A., and McKnight, J. Glucose-mediated functional alterations in resistance arteries isolated from patients with IDDM. *Diabetologia*, **42**, S1, 1225.

Unpublished Presentations to Societies.

- Endothelial and vascular smooth muscle cell function in isolated human diabetic resistance arteries. McIntyre, C.A., Williams, B.C., McKnight, J.A., Lindsay, R.M., Elliot, A., Walker, B.R., Andrews, R.C and Hadoke, P.W.F. Scottish Cardiovascular Forum, Glasgow, 1997 (Oral).
- Endothelial and vascular smooth muscle cell function in resistance arteries isolated from patients with insulin-dependent diabetes mellitus. McIntyre, C.A., Hadoke, P.W.F, Williams, B.C., Lindsay, R.M., Elliot, A. and McKnight, J.A. Scottish Cardiovascular Forum, Edinburgh, 1998 (Poster).
- Impaired receptor-independent vasoconstriction in resistance arteries isolated from patients with non insulin-dependent diabetes mellitus. McIntyre, C.A., Williams, B.C., Andrews, R.C., McKnight, J.A., Walker, B.R. and Hadoke, P.W.F. Scottish Cardiovascular Forum, Edinburgh, 1998 (Poster).
- Glucose-mediated functional alterations in resistance arteries isolated from patients with IDDM. Hadoke,P.W.F., McIntyre, C.A., Jones, G.C., Williams, B.C., Elliot, A., and McKnight, J. Scottish Cardiovascular Forum, Dundee, 2000 (Poster).

CHAPTER 1

INTRODUCTION

The development of diabetes mellitus results in vascular damage which underlies many of the major complications (eg. atherosclerosis, ulceration, peripheral vascular disease, retinopathy, hypertension) associated with this condition. These complications contribute significantly to the morbidity and mortality caused by diabetes. Consequently, they have a significant impact on patient suffering and health care expenditure world-wide. The cause of diabetic vascular disease, however, is not understood, despite extensive investigations in animals and man.

The aim of the research described in this thesis is to assess the effects of diabetes on human microvascular function. Therefore, in order to gain a better understanding of the vascular alterations that may occur in diabetes, it is first necessary to describe normal vascular structure and function. The first part of the introduction will describe the architecture of the vessel wall and the regulation of vascular smooth muscle cell relaxation and contraction. As endothelial cell dysfunction is thought to be central to vascular abnormalities in diabetes, this section will focus on the endothelium-derived relaxing factors (nitric oxide (NO), prostacyclin (PGI₂) and endothelium-derived hyperpolarizing factor (EDHF)) and the endothelium-derived contracting factor (EDCF), endothelin-1 (ET-1). The second part of the introduction will concentrate on the pathology of diabetes mellitus and discuss the mechanisms that may contribute to the altered vascular function in this condition.

1.1. Structure of the vessel wall

With the exception of the capillaries, the vascular wall is composed of three morphologically distinct layers: an inner tunica intima, a central tunica media and an outer tunica adventitia (Figure 1.1).

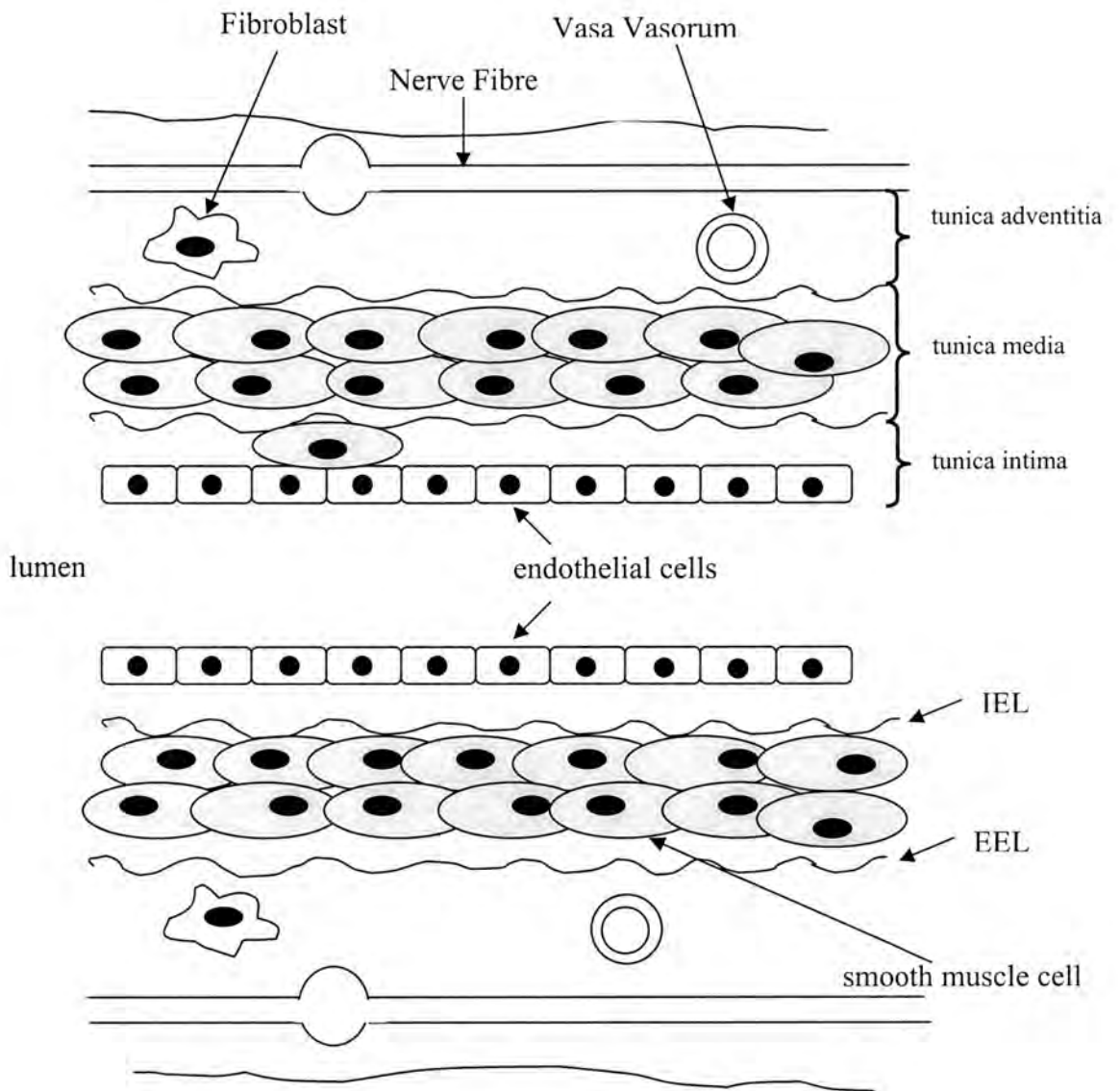


Figure 1.1. Schematic longitudinal section of a small artery showing the three layers of the vascular wall. IEL, internal elastic lamina; EEL, external elastic lamina.

1.1.1. *Adventitia*

The tunica adventitia is the outermost layer of the vascular wall and consists of dense fibroelastic tissue without smooth muscle cells. It harbours nutrient vessels (vasa vasorum) of the vascular wall, nerves and fibroblasts. Its function is to confer rigidity on the vessel wall, carry nutrients to the smooth muscle cells of the media and add stability by connecting the blood vessel to its surrounding tissues.

1.1.2. *Media*

The tunica media, the middle layer of the vascular wall, consists of smooth muscle cells surrounded by elastic sheets (laminae), collagen, and a network of elastic fibrils and proteoglycans (mucopolysaccharides). It is separated from the intima and the adventitia by the internal (IEL) and external (EEL) elastic laminae, respectively. The medial smooth muscle cells provide the vessel with the ability to contract or relax in response to a variety of stimuli.

1.1.3. *Intima*

The intima is the innermost layer of all blood vessels, and is in direct contact with the flowing blood. It consists of (1) a continuous monolayer of endothelial cells lining the vascular wall, (2) a subendothelial layer composed of collagenous bundles, elastic fibrils, smooth muscle cells and some fibroblasts and (3) the IEL (Ross & Glomsett, 1976; Badimon *et al.*, 1993). The composition of the intima varies between blood vessels. For example, the sub-endothelial layer is present in some elastic (e.g. aorta) and muscular (e.g. coronary) arteries, whilst in others the intima consists of only endothelial cells and basal lamina.

The vascular endothelium consists of a continuous, obligate, monolayer of squamous cells lining the luminal surface of blood vessels. Until the 1980s, it was regarded as an inert layer acting as a barrier between the blood and the procoagulant contents of the vessel wall. Since the pioneering work of Furchgott and Zawadzki (1980), however, it has become apparent that the endothelium has a central role in the control of a variety of physiological processes, including; inflammation, coagulation, smooth muscle cell tone and smooth muscle cell proliferation. (Belloni *et al.*, 1992; Ross, 1993 (a&b)).

1.2. The Influence of the Endothelium on Vascular Tone

The vascular endothelium plays a central role in the regulation of blood flow and vascular tone through the synthesis of several potent vasoactive substances. These substances include the vasodilators nitric oxide (NO; Furchgott & Zawadzki, 1980), prostacyclin (PGI₂, Moncada *et al.*, 1976) and endothelium-derived hyperpolarizing factor (EDHF; Bolton *et al.*, 1984) and the vasoconstrictors endothelin-1 (ET-1; Yanagisawa *et al.*, 1988), angiotensin II (AII; Webb & Cockcroft, 1990), and thromboxane A₂ (TxA₂; Auch-Schwelk *et al.*, 1990).

1.2.1. Endothelium-derived relaxing factors

1.2.1.1. Nitric oxide

The demonstration by Furchgott and Zawadzki (1980) that acetylcholine (ACh)-mediated relaxation of isolated arteries was dependent upon an intact endothelium led to the proposal of the existence of an endothelium-derived relaxing factor (EDRF; Furchgott *et al.*, 1984). EDRF was shown to be a diffusible substance that

was eventually identified as NO (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). NO has a half-life of a few seconds and is rapidly oxidised to nitrate by oxygenated haemoglobin, molecular oxygen and superoxide anions, before being excreted in the urine (Moncada & Higgs, 1983; Wennmalm *et al.*, 1993). As well as being a potent vasodilator, endothelium-derived NO has anti-atherogenic properties, including decreasing platelet and leucocyte adhesion to the endothelium and inhibiting vascular smooth muscle cell migration (Moncada *et al.*, 1991).

NO is synthesised within the vascular endothelium by the enzyme nitric oxide synthase (NOS) which converts the amino acid, L-arginine (Palmer *et al.*, 1988a; 1988b) into L-citrulline and NO (Figure 1.2). NOS is an NADPH-dependent dioxygenase which has at least four isoforms, including: a constitutive Ca^{2+} /calmodulin-dependent NOS (eNOS or NOS III), which is mainly present in endothelial cells, and a Ca^{2+} -independent inducible NOS (iNOS or NOS II), which is found in cytokine-activated cells (predominantly macrophages and smooth muscle cells, but also in endothelial cells). Both isoforms require NADPH, FAD and FMN as cofactors and tetrahydrobiopterin potentiates their activity (Forstermann *et al.*, 1991).

NO released under physiological conditions contributes to the regulation of blood flow in healthy individuals (Vallance *et al.*, 1989). Under basal conditions, NO is released spontaneously from the endothelium, in addition, NO synthesis can be stimulated by a number of physical and biochemical stimuli. These activate endothelial NOS through a rise in the concentration of calcium within the endothelial

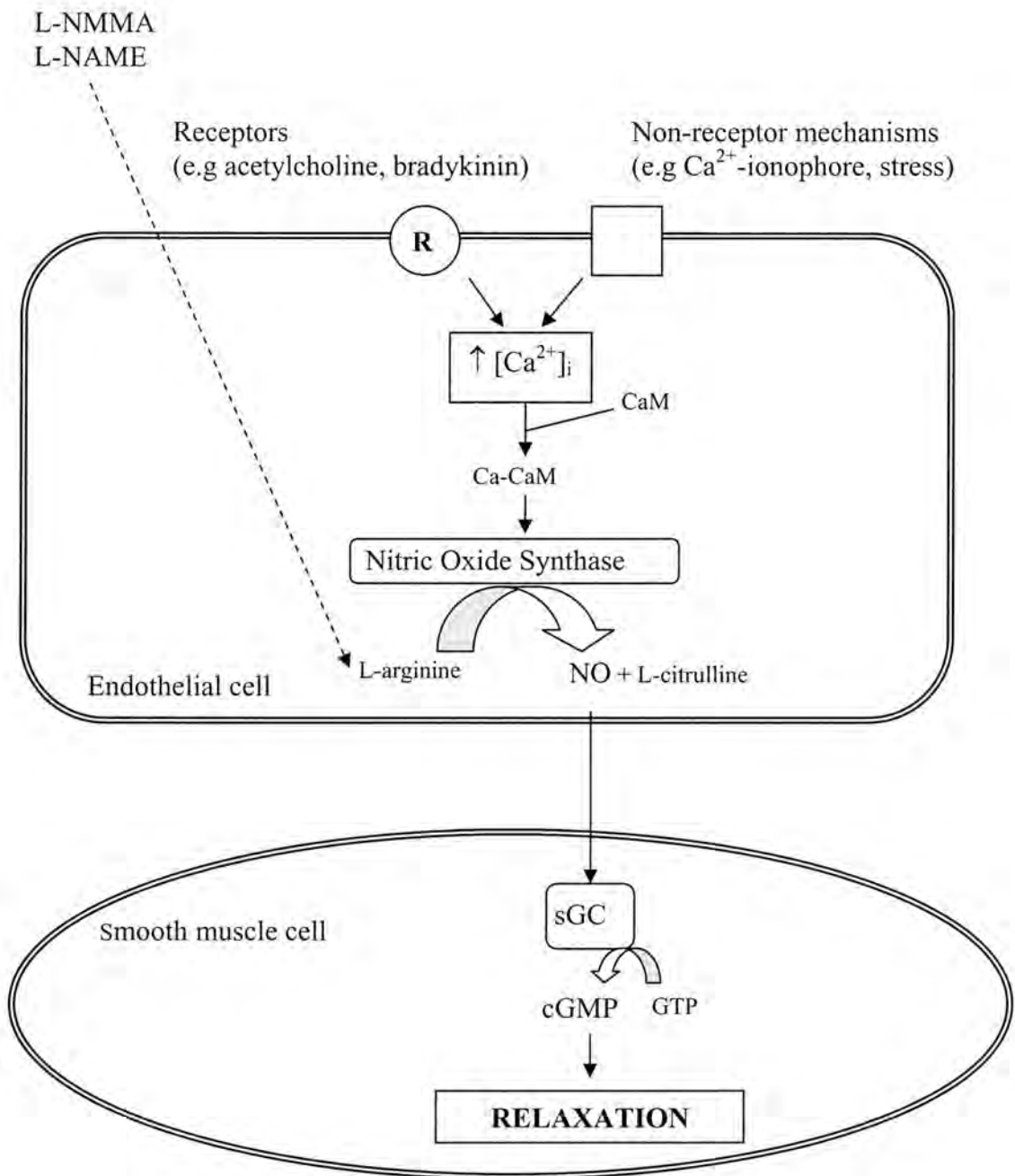


Figure 1.2. Generation of nitric oxide (NO) in the vascular endothelium. NO is produced from the enzymatic conversion of L-arginine to L-citrulline by NO synthase (NOS). After diffusing to the underlying smooth muscle, NO activates soluble guanylate cyclase (sGC), leading to the accumulation of cyclic guanosine monophosphate (cGMP). cGMP-dependent protein kinases then mediate smooth muscle relaxation. Synthesis of NO can be inhibited (broken line) by analogues of L-arginine, such as N^G -monomethyl L-arginine (L-NMMA) and N^G -nitro-L-arginine methyl ester (L-NAME). R, receptor, CaM, calmodulin.

cell. Such stimuli include shear stress, pulsatile flow and hypoxia (Lamotagne *et al.*, 1992; Pohl & Busse, (1989), as well as receptor-dependent agonists (e.g. acetylcholine, bradykinin) and (receptor independent) calcium ionophores, such as A23187.

Once formed, NO diffuses to the underlying smooth muscle, where it stimulates soluble guanylate cyclase through which it causes relaxation by reducing intracellular calcium concentrations (See Chapter 1.3.2). Analogues of L-arginine, such as L-N^G monomethyl arginine (L-NMMA), act as inhibitors of NOS and have been found to cause vasoconstriction (Vallance *et al.*, 1989), indicating that NO exerts a tonic dilator action opposing constrictor influences in the arterial circulation. Most veins produce little, if any, NO under resting conditions and are very sensitive to exogenous NO in the form of nitrovasodilators (Vallance *et al.*, 1989).

1.2.1.2. Prostacyclin

Prostacyclin, or prostaglandin I₂ (PGI₂), was first identified in 1976 by Moncada and colleagues (Moncada *et al.*, 1976). It is generated predominantly in endothelial cells (although it can also be formed in the smooth muscle) where it is produced from arachidonic acid through the action of the enzymes cyclooxygenase and prostacyclin synthase. Once bound to its receptor (see review by Pierce *et al.*, 1995), the vasodilatory and anti-aggregatory effects of PGI₂ are mediated through a rise in intracellular cyclic AMP concentrations (see Chapter 1.3.2) in smooth muscle cells and platelets, respectively (Tateson *et al.*, 1997). Pulsatile flow and vasoactive mediators such as bradykinin and thrombin stimulate PGI₂ synthesis (Piper & Vane,

1977). It appears that arteries can synthesise three to ten times more PGI₂ than veins (Skidgel & Printz, 1978), and larger vessels produce more PGI₂ than the microvasculature (MacIntyre *et al.*, 1978).

1.2.1.3. Endothelium-derived hyperpolarising factor.

In some vessels, particularly those with a small diameter (Shimokawa *et al.*, 1996), a component of the endothelium-dependent relaxation is insensitive to NO synthase and cyclooxygenase inhibition (Nagao *et al.*, 1992; Brandes *et al.*, 1997). This NO- and prostanoid-independent component appears to be mediated by hyperpolarization of the vascular smooth muscle cells (Brayden, 1990), suggesting the existence of a distinct endothelium-derived hyperpolarizing factor (EDHF) (Taylor & Weston, 1988; Feletou & Vanhoutte, 1997).

The identity of EDHF has yet to be confirmed, although activity of this factor has been attributed to epoxyeicosatrienoic acids (Hecker *et al.*, 1994), endocannabinoids (Randall *et al.*, 1996), hydrogen peroxide (Matoba *et al.*, 2000), the presence of myoendothelial gap junctions (Chaytoe *et al.*, 1998) and release of K⁺ into the myoendothelial space (Edwards *et al.*, 1998).

1.2.2. *Endothelium-derived vasoconstrictors*

Although the endothelium can release several distinct vasoconstrictors (ET-1, TxA₂, AII) it is endothelin (ET) that has most relevance to the work described in this thesis.

1.2.2.1. Endothelin

Endothelin was originally identified as a 21 amino acid vasoconstrictor peptide in the cultured supernatant of porcine aortic endothelial cells (Yanagisawa *et al.*, 1988). ET exists as a family of three isopeptides: ET-1, the isoform originally identified by Yanagisawa *et al.*, (1988), and ET-2 and ET-3, which are structurally and pharmacologically distinct (Inoue *et al.*, 1989). ET-1 is the major form produced by vascular endothelial cells in humans, and is generated in response to hypoxia, vascular shear stress, and a range of other vasoactive mediators (Yanagisawa *et al.*, 1988). It is synthesised by transcription and translation of a 212-amino acid prepropeptide (prepro ET) which is proteolytically cleaved to produce the 38 amino acid intermediate, big endothelin-1 (big ET-1). This is subsequently converted to mature ET-1 by ET-converting enzyme (ECE).

ET-1 binds to specific G-protein-coupled cell surface receptors located both on endothelial cells and vascular smooth muscle cells. There are at least two distinct receptor subtypes, designated ET_A and ET_B. ET_A and ET_B receptors are expressed on vascular smooth muscle cells where they mediate vasoconstriction. The ET_B receptor is also expressed on endothelial cells where it mediates vasodilatation. The primary target of ET-1 is the vasculature where it evokes transient vasodilatation mediated by endothelial ET_B receptors, followed by slow-onset and sustained contraction mediated by ET_A and ET_B receptors located on vascular smooth muscle cells. The functional response to ET-1 varies between vessels and species due to differences in distribution and expression of these two receptor subtypes (Haynes & Webb, 1993).

1.3. Regulation of vascular tone

1.3.1. Smooth muscle cell contraction

The intracellular concentration of free calcium ($[Ca^{2+}]_i$) is a primary determinant of contractile activity in smooth muscle cells (Figure 1.3) (Filo *et al.*, 1965). Increases in $[Ca^{2+}]_i$ levels in smooth muscle cells triggers the activation of Ca^{2+} -calmodulin-dependent myosin light chain kinase (MLCK), which in turn activates myosin light chain kinase to phosphorylate the light chain sub-unit of myosin, resulting in an increase in actin-activated myosin Mg-ATPase activity (Dabrowska *et al.*, 1978). This enables myosin to interact with actin, thereby initiating the contractile process (Cohen, 1993).

Smooth muscle $[Ca^{2+}]_i$ increases during contraction either as a consequence of influx of Ca^{2+} from the extracellular space or release of Ca^{2+} from the sarcoplasmic reticulum. Influx of Ca^{2+} from the extracellular space occurs through voltage-operated channels (VOC), receptor-operated channels (ROC) and non-selective cation channels (NSCC) and by a reversed Na^+/Ca^{2+} exchange mechanism (Vanhoutte, 1990). Ca^{2+} release from intracellular stores proceeds through inositol 1,4,5-trisphosphate (IP_3)-regulated channels or by Ca^{2+} -induced Ca^{2+} release, generally via ryanodine receptor-regulated channels (Herrmann-Frank *et al.*, 1991).

Voltage-operated channels are selectively permeable to Ca^{2+} and are activated by membrane depolarization; repolarization and hyperpolarization result in deactivation of these channels. Depolarization can be induced by high concentrations of

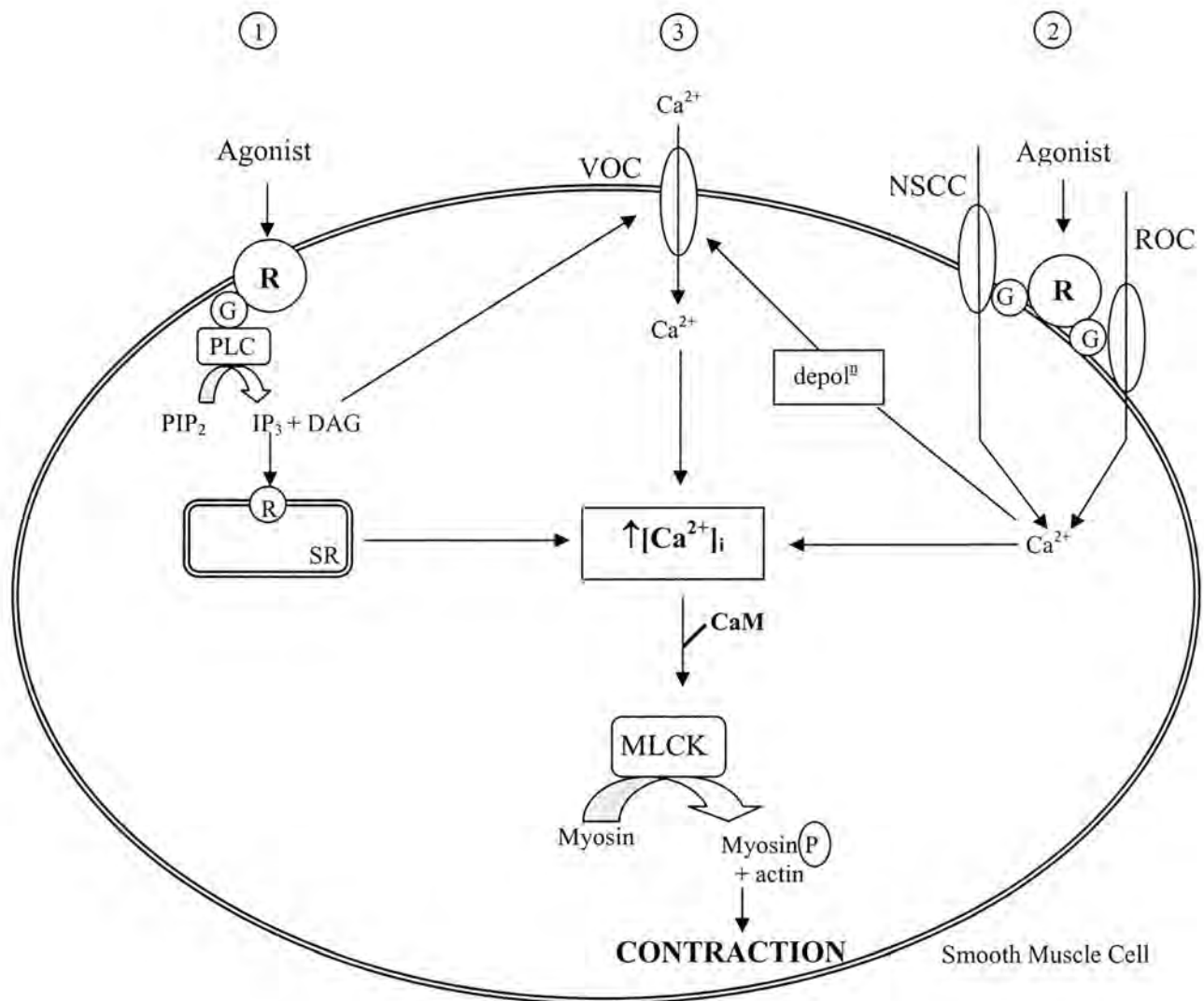


Figure 1.3. Mechanisms of contraction in vascular smooth muscle cells. Contraction occurs when intracellular Ca^{2+} and calmodulin combine to activate phosphorylation of myosin light chain kinase (MLCK), leading to actin-myosin cycling which generates active tension. Vasoconstrictors can act by increasing $[Ca^{2+}]_i$ in various ways (1) stimulating receptors coupled to phospholipase C (PLC), which hydrolyses phosphatidylinositol bisphosphate (PIP_2), leading to inositol triphosphate (IP_3) and diacylglycerol (DAG) production and the release of stored Ca^{2+} from the sarcoplasmic reticulum (SR). (2) Stimulating receptor-operated channels (ROC) and non-specific cation channels (NSCC), which allow Ca^{2+} entry and also cause depolarization. (3) Stimulating voltage-operated Ca^{2+} channels (VOC), which open in response to depolarization and may also be facilitated indirectly by agonists. Release of Ca^{2+} from SR produces a transitory contraction, whilst extracellular Ca^{2+} is required for maintenance of a sustained response.

extracellular K^+ or by agonist-induced activation of receptor-operated channels (Walsh, 1994).

The stimulation of various receptors (e.g. adrenergic, purinergic, cholinergic, histaminergic, serotonergic, endothelin, stretch) by agonists can increase $[Ca^{2+}]_i$ via two mechanisms. Firstly, G-protein-coupled receptor stimulation activates phospholipase C (PLC), which, in turn, hydrolyses phosphatidylinositol 4,5 bisphosphate (PIP_2), leading to the generation of two intracellular second messengers, inositol triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 diffuses to the sarcoplasmic reticulum, where it induces the release of intracellular Ca^{2+} . DAG production leads to the stimulation of protein kinase C (PKC), which induces opening of Ca^{2+} channels. The second receptor-dependent mechanism involves activation of either receptor-operated G-protein-dependent Ca^{2+} channels or receptor-operated non-specific cation channels (Reviewed in Rembold, 1992; Cohen, 1993).

The contractile response of vascular smooth muscle occurs in two phases, (1) the initial (phasic) component and (2) the sustained (tonic) component. It has been demonstrated that the initial (phasic) contraction is produced by the release of intracellular Ca^{2+} , while the sustained (tonic) contraction is induced by the influx of Ca^{2+} via activation of membrane channels. As vascular smooth muscle can remain in a contracted state for prolonged periods of time, the contraction is generally tonic in nature and therefore Ca^{2+} influx, rather than release, is the most important for maintaining tone (Bosnjak, 1993).

1.3.2. Smooth muscle cell relaxation

Vascular smooth muscle relaxes when $[Ca^{2+}]_i$ falls below a threshold level. The removal of Ca^{2+} from the cytoplasm is controlled by a combination of Ca^{2+} accumulation into the sarcoplasmic reticulum and extrusion from the cell across the plasma membrane (Figure 1.4). The major route of Ca^{2+} removal is the uptake of Ca^{2+} into the sarcoplasmic reticulum, which occurs via a Ca^{2+} , Mg^{2+} -ATPase pump that undergoes Ca^{2+} -dependent phosphorylation. The activity of the pump is regulated by phospholamban, a protein localised to the sarcoplasmic reticulum, as well as cyclic nucleotide-dependent phosphorylation (Tada & Katz, 1982). The plasma membrane contains a Ca^{2+} , Mg^{2+} -ATPase pump, distinct from that found in the sarcoplasmic reticulum, which extrudes Ca^{2+} from the cell. This ATPase is stimulated by calmodulin when $[Ca^{2+}]_i$ rises (Missiaen *et al.*, 1992). Smooth muscle membranes may also contain a Na^+ , Ca^{2+} exchanger, which may contribute to Ca^{2+} extrusion in some blood vessels. The relative contribution of each of these processes, however, may vary among different vascular beds (Reviewed in Gurney, 1994)

Since the opening of Ca^{2+} channels requires membrane depolarization, agents that produce hyperpolarization will cause Ca^{2+} channels to close, which in turn will reduce Ca^{2+} influx and promote muscle relaxation (Nelson *et al.*, 1990). The endothelium of a number of arteries releases a substance that evokes hyperpolarization of vascular smooth muscle cells and relaxation. Although NO can cause hyperpolarization of vascular smooth muscle cells (Plane *et al.*, 1996), endothelium-dependent hyperpolarizations are mediated mainly by a distinct NO-independent EDHF (Bolton *et al.*, 1984).

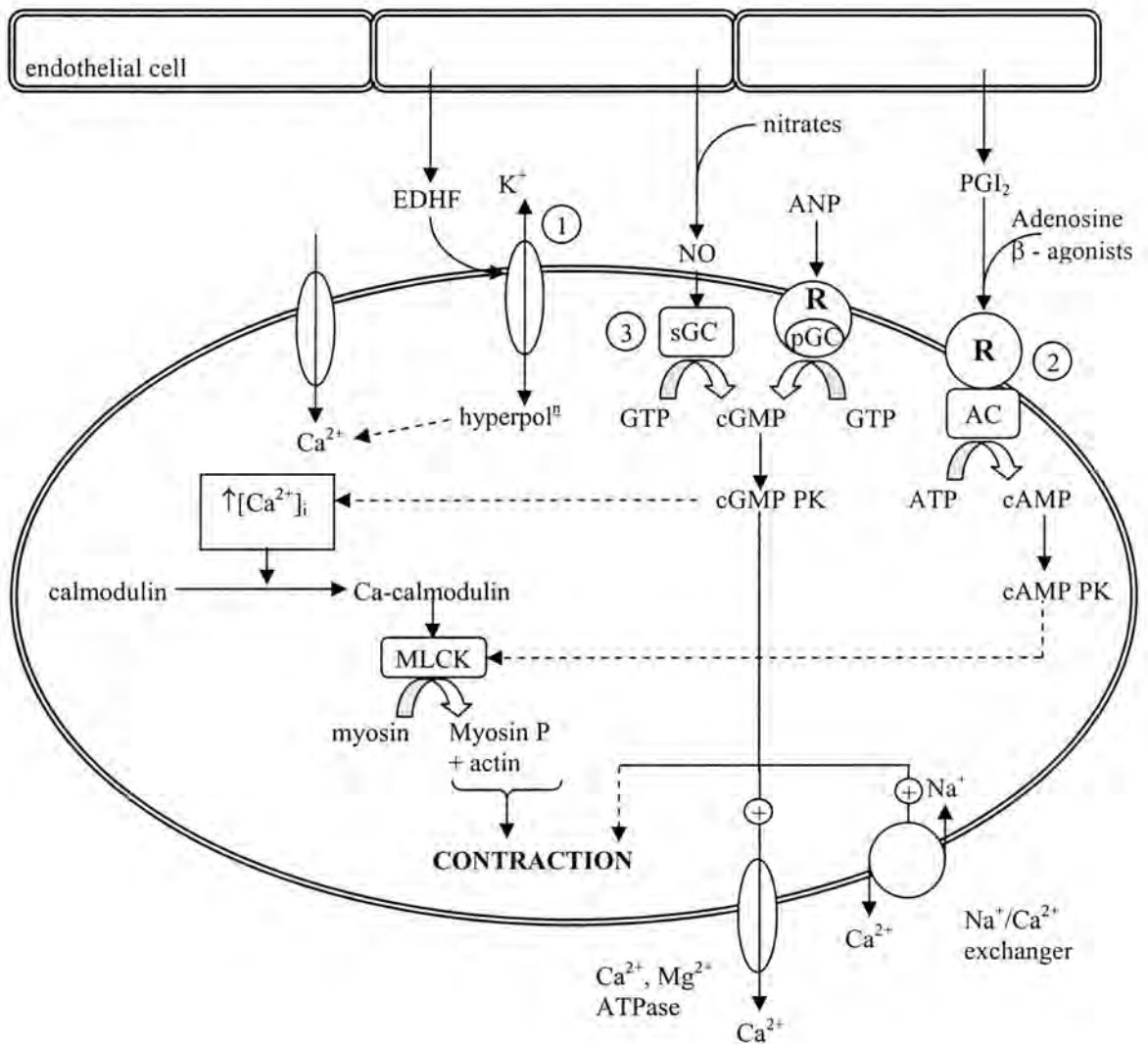


Figure 1.4. Mechanisms of agonist-induced relaxation in vascular smooth muscle cells. Relaxation occurs when $[Ca^{2+}]_i$ falls below a threshold level. Vasodilators may work by reducing $[Ca^{2+}]_i$ or directly on the contractile machinery and can be achieved by: (1) opening of K⁺ channels, causing hyperpolarization, and thus preventing voltage-gated Ca²⁺ channels from opening. (2) receptors coupled to adenylate cyclase (AC), activation of which cause increased cAMP production. This acts via a cAMP-dependent protein kinase (cAMP PK) and myosin light chain kinase (MLCK) to inhibit contraction. (3) stimulation of soluble (sGC) or particulate (pGC) guanylate cyclase increases cGMP formation. This acts via a cGMP-dependent protein kinase (cGMP PK) to cause relaxation by stimulating both Ca²⁺ uptake into the SR (via a phospholamban-mediated Ca²⁺Mg²⁺ ATPase) and extrusion of Ca²⁺ across the cell membrane via a calmodulin-sensitive Ca²⁺Mg²⁺ ATPase pump and Na⁺Ca²⁺ exchange mechanism. It also reduces the Ca²⁺ sensitivity of contractile proteins. ANP, atrial natriuretic peptide; EDHF, endothelium-derived hyperpolarising factor; NO, nitric oxide; PGI₂, prostacyclin.

Relaxation induced by NO and NO releasing drugs is preceded by an elevation of intracellular cGMP levels in vascular smooth muscle. NO and NO-releasing compounds such as sodium nitroprusside (SNP), activate soluble guanylate cyclase (sGC), which stimulates the production of cGMP from GTP (Rapaport *et al.*, 1983). The effects of cGMP are mediated through cGMP-dependent protein kinase, which is known to phosphorylate smooth muscle cell proteins. The main effect of cGMP-dependent vasodilators is to lower $[Ca^{2+}]_i$, which can be achieved by a number of different mechanisms (reviewed in Gurney, 1994; Walsh, 1993):- (1) inhibition of voltage- and receptor-operated channels, (2) hyperpolarization, (3) inhibition of Ca^{2+} release from the sarcoplasmic reticulum, by inhibiting PLC and the production of IP_3 (4) stimulation of both Ca^{2+} uptake by the sarcoplasmic reticulum and membrane Ca^{2+} , Mg^{2+} -ATPase, which pumps Ca^{2+} out of the cell and (5) reduction in sensitivity of contractile proteins to Ca^{2+} (Figure 1.4). Since nitrovasodilators inhibit agonist-induced contractions more effectively than depolarization-induced contractions, the major pathway for relaxation appears to be inhibition of receptor-dependent contractions. Receptor-operated Ca^{2+} channels are inhibited, decreasing Ca^{2+} influx, and cGMP-dependent protein kinase phosphorylates phospholamban, an endogenous regulator of the sarcoplasmic reticulum Ca^{2+} , Mg^{2+} -ATPase, which enhances uptake of Ca^{2+} by the sarcoplasmic reticulum. In addition, cGMP may inhibit agonist-induced release of Ca^{2+} by the sarcoplasmic reticulum by inhibition of PLC and the production of IP_3 . Several additional mechanisms may also explain cGMP-dependent vasodilation. These include decreased sensitivity of contractile proteins to Ca^{2+} , by a mechanism linked to cGMP-dependent phosphorylation of MLCK, and stimulation

of a membrane Ca^{2+} , Mg^{2+} -ATPase pump which pumps Ca^{2+} out of the smooth muscle cell.

1.4. Diabetes Mellitus

Diabetes mellitus is a syndrome with characteristic hyperglycaemia, which results from a deficiency (either relative or absolute) in insulin (World Health Organisation (WHO), 1985). The clinical diagnosis of diabetes is often indicated by the presence of symptoms such as polyuria, polydipsia, blurring of vision and unexplained weight loss, and in severe cases, drowsiness and coma and is confirmed by measurement of abnormal hyperglycaemia. The ranges of blood glucose indicative of diabetes mellitus are random venous plasma glucose $\geq 11.1\text{mmol/l}$ or fasting plasma glucose $\geq 7.0\text{mmol/l}$ or plasma glucose $\geq 11.1\text{mmol/l}$ two hours after a 75g oral glucose load (the oral glucose tolerance test) (WHO, 1999).

There are two distinct types of primary diabetes: Type 1 (previously referred to as insulin-dependent diabetes mellitus, IDDM), and Type 2 (previously referred to as non-insulin-dependent diabetes mellitus, NIDDM). Although the exact aetiology for both types of primary diabetes mellitus is improperly understood, it is clear that interactions between environmental and genetic factors determine both whether an individual will develop diabetes and the timing of disease onset (Rossini *et al.*, 1993).

1.4.1. Type 1 Diabetes Mellitus

Type 1 diabetes mellitus usually appears before the age of 40, often in childhood and adolescence. The onset of symptoms may be abrupt, with thirst, excessive urination,

increased appetite, and weight loss developing over a period of days to weeks. Characteristically, a total deficiency in pancreatic β -cells in Type 1 diabetes leads to an absolute deficiency of insulin and elevated glucagon levels. This type of diabetes is unresponsive to treatment with oral antidiabetic drugs and, therefore, insulin therapy is always required for patients with Type 1 diabetes (Frier *et al.*, 1999).

Type 1 diabetes has a polygenic inheritance with 33% of cases having a genetic component (Atkinson & Eisenbarth, 2001). However, studies in identical twins have shown concordance lower than 40%, indicating that environmental factors (e.g. viruses, diet, stress) play an important role in disease development (Olmos *et al.*, 1988). There is also evidence that Type 1 diabetes is a chronic, T-cell mediated, autoimmune disease with destruction of insulin secreting cells in the pancreas occurring over many years (Atkinson & Eisenbarth, 2001).

1.4.2. *Type 2 Diabetes Mellitus*

Unlike Type 1, Type 2 diabetes mellitus usually occurs in middle or later life (although earlier forms, such as maturity onset diabetes of the young (MODY) are known (Guazzarotti *et al.*, 1999)). It is commonly diagnosed in obese, insulin resistant patients but these factors only cause diabetes if accompanied by impaired β cell function. Symptoms begin more gradually than in Type 1 (developing over months-years) and, in direct contrast, plasma insulin levels are elevated. Patients often present with chronic fatigue and malaise but much Type 2 diabetes is undetected and it is often only diagnosed serendipitously (Frier *et al.*, 1999).

Genetics are more important in the development of Type 2 diabetes with almost 100% concordance in identical twins (some forms, including MODY, result from a single gene defect but these account for <5% of cases (Hattersley, 1998)). Most cases are multi-factorial, resulting from an interaction between environmental and genetic factors. Environmental factors include lifestyle (over-eating, obesity, under-activity), malnutrition *in utero* (inverse relationship between birth weight and Type 2 diabetes), age (more than 70% of cases occur in individuals over the age of 50) and pregnancy. Obesity is a major risk factor for the insulin resistance and higher plasma insulin concentrations seen before and after the development of hyperglycaemia (Truglia *et al.*, 1985). It is for this reason that although Type 2 diabetes is responsive to treatment with oral anti-diabetic drugs, many patients are treatable with diet and exercise alone. However, in some cases where hyperglycaemic control is not achievable, a combination of oral anti-diabetic drugs with insulin is useful for the treatment of Type 2 diabetes (Simpson *et al.*, 1990).

1.4.3. *The Pathophysiology of Diabetes Mellitus.*

The tight regulation of blood glucose in the range 3.5-6.5mmol/ l is important for normal metabolism, especially for the brain which uses glucose as its principal metabolic fuel. Blood glucose concentrations reflect a balance between glucose entering the circulation (from the liver as a result of gluconeogenesis and glycogenolysis but also from the gut following meals) and uptake in peripheral tissues (particularly skeletal muscle). Insulin, which is secreted into the portal blood from pancreatic β -cells, reduces blood glucose by suppressing hepatic glucose production and stimulating uptake (mainly by skeletal muscle and fat). Insulin also

prevents catabolism of fats (non-esterified fatty acids (NEFAs) and glycerol) by stimulating lipogenesis and inhibiting lipolysis in the adipocytes and liver. Lipolysis liberates NEFAs which provide energy for gluconeogenesis, producing ketone bodies as a by-product (Frier *et al.*, 1999). Consequently, in the face of insulin deficiency, inhibition of lipolysis is reduced, resulting in hyperketonaemia,

Hyperglycaemia develops as a consequence of absolute (Type 1 diabetes) or relative (Type 2 diabetes) insulin deficiency. This high concentration of blood glucose, and other biochemical abnormalities, results from a deficiency in pancreatic β -cells and/or from an impaired sensitivity to insulin in target cells (World Health Organisation (WHO) Study Group, 1985).

1.4.4. *Complications of Diabetes Mellitus*

Lack of insulin interferes with carbohydrate, protein and fat metabolism and causes a severe disturbance of water and electrolyte balance (Frier *et al.*, 1999). Death can occur from acute metabolic decompensation whereas chronic metabolic abnormalities are often associated with permanent and irreversible changes in the function and structure of cells. Vascular cells are particularly susceptible to these changes and this results in the complications of diabetes which characteristically affect the eye, the kidney and the nervous system (Kohner *et al.*, 1995). Diabetes is associated with an excess mortality of between 3-5 times the non-diabetic population, mainly as a consequence of higher rates of vascular disease (Jarrett, 1989).

1.4.4.1. Macrovascular Disease.

Diabetes is an important and independent risk factor for the development of coronary artery disease and peripheral vascular disease, which are the principal causes of death and disability in patients with this condition (Head & Fuller, 1990). Atherogenesis follows a pattern similar to that in the non-diabetic patient but lesions develop earlier and are both more extensive and more severe. Diabetes enhances the effect of other risk factors (smoking, hypertension, hypercholesterolaemia) whilst hyperinsulinaemia may raise arterial blood pressure and may also promote atherogenic changes in blood lipids and coagulability (Morrish *et al.*, 1991).

1.4.4.2. Microvascular Disease.

Small blood vessel disease (diabetic microangiopathy) is a specific complication of diabetes. Diabetes is associated with microvascular complications in the retina, kidney and peripheral nervous system, which contribute significantly to morbidity and mortality (Eurodiab IDDM Complications Study Group, 1994). The classical features of diabetic microangiopathy, a marked thickening of the capillary basement membrane and an increase in vascular permeability, are found in all vascular beds (Tooke, 1996). The imposition of tissue and organ specific factors on this general vascular injury lead to the development of the clinical symptoms: retinopathy, nephropathy and neuropathy.

1.4.4.2.1. Diabetic Retinopathy

In the retina, the vascular pathologies are found mostly in the microvessels. Among the earliest detectable histological changes is a loss of retinal pericytes (Chibber *et*

al., 1994; Kohner *et al.*, 1995). Other changes include increased capillary diameter, basement membrane thickening, changes in retinal blood flow, increased vascular permeability and formation of microaneurysms, all of which could be consequences of pericyte loss (Parving *et al.*, 1983; Kohner *et al.*, 1995). Once formed, the abnormal blood vessel appears to lose its ability to regulate blood flow; the capillaries in turn lose their ability to receive blood, which leads to formation of ghost capillaries (Kohner *et al.*, 1995). The increased haemorrhages and exudates combine with the formation of ghost vessels to produce large areas of anoxia, leading to the more serious stage of diabetic retinopathy, proliferative retinopathy (Aiello *et al.*, 1998).

Proliferative retinopathy is characterised by neovascularisation of retinal capillaries both intraretinally and in the vitreous; this leads to increased risk of bleeding and, with healing, to the formation of fibrosis in the retina that enhances the potential for retinal detachment and visual loss. Overall, diabetic retinopathy is the most common cause of blindness in adults aged 20-74 years (American Diabetes Association, 2002(a)).

1.4.4.2.2 Diabetic Nephropathy

Diabetic nephropathy is a significant cause of morbidity and mortality: renal failure accounts for 40-50% of deaths in those developing diabetes before age 15 and is among the most common causes of end-stage renal failure (American Diabetes Association, 2002(b)). Approximately 30% of patients with type 1 diabetes will develop nephropathy within 20 years and its treatment is difficult. In the kidney,

increased renal blood flow contributes to the glomerular hyperfiltration and hypertension which results in diabetic nephropathy (Hostetter *et al.*, 1982), diagnosis of which is indicated by the development of microalbuminuria and proteinuria.

1.4.4.2.3. Diabetic Neuropathy

In diabetic neuropathy (Stevens *et al.*, 1995; Malik *et al.*, 1993) (a common, early complication affecting ~30% of patients), capillary lesions may be found in both afferent and efferent, somatic and autonomic nerves. The manifestations of this complication include loss of sensation, paresthesia (abnormal sensation), weakness, difficulty in walking and autonomic dysfunction (e.g. urinary retention, impotence and orthostatic hypotension).

1.5. Evidence of Vascular Dysfunction in Diabetes Mellitus

The pathogenesis of the vascular complications of diabetes mellitus is not completely understood. However, there is considerable evidence to indicate that both macrovascular and microvascular complications are the result of impaired function of vascular cells (Tooke, 1996). The “haemodynamic hypothesis” of the pathogenesis of diabetic microangiopathy in type 1 diabetes argues that in the early stages of diabetes microvascular pressure and flow are increased, resulting in an injury response in the microvasculature with a concomitant thickening of the basement membrane. This in turn limits the vasodilatory capacity of the microvasculature impairing maximal hyperaemia as well as interfering with the ability of the microcirculation to autoregulate (Parving *et al.*, 1983).

Early in the course of type 1 diabetes there is an increase in retinal, renal, skeletal muscle and skin blood flow, which is associated with poor glycaemic control (reviewed Tooke, 1986). Nailfold capillary pressure is increased early during the course of diabetes and is related to the degree of hyperglycaemia (Sandeman *et al.*, 1992). Furthermore, capillary pressure is elevated in type 1 patients with microalbuminuria (Shore *et al.*, 1992), compared with age- and sex-matched patients of similar disease duration and glycaemic control without clinical evidence of microvascular complications.

In contrast to type 1 diabetes, the major abnormality of microvascular function found in the skin of patients with type 2 diabetes is a marked impairment of microvascular vasodilatation. This is present at the time of diagnosis (Sandeman *et al.*, 1991) and may be related to the effects of hyperinsulinaemia in the pre-diabetic state (Jaap & Tooke, 1995). Despite the marked changes described in type 1 diabetes, skin nailfold capillary pressure appears to be normal in normotensive patients with type 2 diabetes (Shore *et al.*, 1994).

The ability to measure human microvascular function directly is crucial in understanding the pathophysiology of diabetic microangiopathy. Results from such studies have provided a pathophysiological framework which suggests that there are differences between the pathogenesis of vascular alterations between the two major forms of diabetes (Tooke, 1995), and this is pertinent to understanding the underlying mechanisms which ascribe a pivotal role for the vascular endothelium.

1.5.1. *Endothelium-Dependent Vascular Responses in Diabetes*

Studies of vascular function in diabetes are complicated by the recognised difficulties of analysing vascular function *in vivo* and isolating tissue from patients for study *in vitro* (Benjamin *et al.*, 1995). Functional investigations of endothelial cell function in diabetic patients have provided conflicting results. The discrepancies between studies could be attributed to variations in a number of factors, including differences in the duration and degree of the diabetic state, degree of acute hyperglycaemia, insulin concentrations, and the presence or absence of diabetic complications.

1.5.2. *Human Studies*

1.5.2.1. *In vivo* studies

In vivo studies of vascular function in diabetes are contradictory, and the nature of the functional alterations remains unclear (Hopfner & Gopalakrishnan, 1999; Porta *et al.*, 1987). Less invasive methods have been employed to determine whether endothelium-dependent vasodilator function is compromised in diabetic subjects. The techniques used include the measurement of forearm blood flow (FBF) using venous occlusion plethysmography (Johnstone *et al.*, 1995), measurement of forearm skin erythrocyte flux to iontophoresis of charged substances using laser doppler perfusion imaging (Morris *et al.*, 1995) and measurement of artery diameter and blood flow velocity, using high-resolution Doppler ultrasound (Zenere *et al.*, 1995).

Venous occlusion plethysmography, which measures total forearm blood flow, is a widely used, safe and accurate technique in which endothelium-dependent vasodilatation of human resistance arteries is assessed by intra-arterial infusion of

muscarinic agonists such as ACh, methacholine or carbachol (Benjamin *et al.*, 1995). In Type 1 diabetes the majority of *in vivo* studies have failed to confirm the defect reported in the isolated subcutaneous arteries (McNally *et al.*, 1994). Responses to infused muscarinic agonists, including ACh, have been no different from that of non-diabetic subjects (Halkin *et al.*, 1991; Calver *et al.*, 1992; Elliott *et al.*, 1993; Smits *et al.*, 1993). However, the NO-mediated component of dilatation was reduced in patients with microalbuminuria (Elliott *et al.*, 1993), and two of the studies reported reduced basal NO release (Calver *et al.*, 1992; Elliott *et al.*, 1993). In patients with Type 2 diabetes, impaired responses to ACh have been observed in the forearm together with blunted responses to NO donors such as GTN and SNP (McVeigh *et al.*, 1992; Watts *et al.*, 1996). These results are challenged, however, by similar investigations demonstrating that endothelium-dependent relaxation is unchanged (Avogaro *et al.*, 1997; Cockcroft *et al.*, 1998) or enhanced (Cipolla *et al.*, 1996) (Tables 1.1; Table 1.2).

The technique of venous occlusion plethysmography has some potential problems, especially when comparing responses between healthy control subjects and patients. Differences in starting conditions, including basal blood flow, arterial pressure and forearm size, can all affect the response to drugs and can result in misleading conclusions being drawn (Benjamin *et al.*, 1995). Furthermore, small alterations in arterial pressure and sympathetic arousal must be controlled for by using the noncannulated arm as an in-built control and by expressing data as percentage change in the of forearm blood flow in the two arms (Benjamin *et al.*, 1995).

Table 1.1. Investigation of human endothelial function in Type I diabetic subjects, studied by venous occlusion plethysmography (VOP), vascular ultrasound (US) and isolated vessel techniques, mainly small vessel myography (SVM).

Endothelium-dependent relaxation	Endothelium-independent relaxations	Method	Disease Duration (yrs)	HbA1c (%)	Reference
Reduced	Reduced (GTN)	US	10±1	7.7±0.2	Zenere <i>et al</i> (1995)
	Reduced (GTN)	US	13±8	9.5±2.2	Clarkson <i>et al</i> (1996)
	Unaltered (SNP)	FBF	14±2	11.9±0.6	Johnstone <i>et al</i> (1993)
	Unaltered (SNP)	FBF	18±2	8.3±0.4	O'Driscoll <i>et al</i> (1997)
	Unaltered (ISDN)	US	12.9±8.4	6.5±1.5	Lekakis <i>et al</i> (1997)
	Unaltered (GTN)	US	26.9±2.0	9.6±0.3	Meeking <i>et al</i> (1999)
Reduced (ACh)	Unaltered (SNP)	SVM	18.1±1.3	9.5±0.5	McNally <i>et al</i> (1994)
Unaltered (BK)	Unaltered (SNP)	FBF	12.0±8.0	N/A	Halkin <i>et al</i> (1991)
Unaltered		FBF	Recent onset	6.7±0.5	Calver <i>et al</i> (1992)
	Unaltered (verapamil)				
	Reduced (SNP)				
	Unaltered (SNP)	FBF	15.1±8.2	9.2±0.9	Smits <i>et al</i> (1993)
	Unaltered (SNP)	FBF	20.7	N/A	Elliot <i>et al</i> (1993)
	Unaltered (GTN)	US	14.9±8	7.9±1.2	Lambert <i>et al</i> (1996)
	Unaltered (SNP)	FBF	18±3	8.6±0.3	Mäkimattila <i>et al</i> (1997)
	Unaltered (GTN)	US	21.5±10.2	8.0±1.1	Enderle <i>et al</i> (1998)
	Unaltered (SNP)	FBF	17	8.98	Huvers <i>et al</i> (1999)
	Reduced (SNP)	FBF	10.8±2.7	9.6±0.7	Khan <i>et al</i> (1996)
	Enhanced (SNP)	SVM	24.9±11.0		Malik <i>et al</i> (1999)

ACh, acetylcholine, BK, bradykinin, FBF, fore-arm blood flow, GTN, glyceryl trinitrate, SNP, sodium nitroprusside, HbA1c, glycosylated haemoglobin.

Table 1.2. Investigation of human endothelial function in Type 2 diabetic subjects, studied by venous occlusion plethysmography (VOP), vascular ultrasound (US) and isolated vessel techniques, mainly small vessel myography (SVM).

Endothelium-dependent relaxation	Endothelium-independent relaxations	Method	Disease Duration (yrs)	HbA1c (%)	Reference
Reduced	Reduced (GTN)	FBF	5.2	9.7	McVeigh <i>et al</i> (1992)
	Reduced (SNP)	US	9.1±1.9	6.5±0.2	Morris <i>et al</i> (1995)
	Reduced (SNP)	FBF	3.6	7.5	Watts <i>et al</i> (1996)
	Unaltered (SNP)	FBF	3.2	7.9±0.7	Ting <i>et al</i> (1996)
	Unaltered (GTN)	US	7.4±6.0	9.1±2.4	Enderle <i>et al</i> (1998)
	Unaltered (SNP)	FBF	Up to 10yrs	10.3±0.7	Hogikyan <i>et al</i> (1998)
Reduced (ACh)	Unaltered (SNP)	FBF	4.6±2.7	6.9±1.4	Gazis <i>et al</i> (1998)
Unaltered (BK)					
Unaltered	Unaltered (SNP)	FBF	N/A	7.0±1.5	Cockcroft <i>et al</i> (1998)
	Unaltered (SNP)	FBF	7±2	8.7±0.6	Avogaro <i>et al</i> (1997)
Unaltered (ACh)	Increased (SNP)	SVM	N/A	N/A	Lawrence <i>et al</i> (1994)
Increased (BK)					
Increased	Unaltered (SNP)	PM	N/A	N/A	Cipolla <i>et al</i> (1996)

ACh, acetylcholine, BK, bradykinin, FBF, fore-arm blood flow, GTN, glyceryl trinitrate, SNP, sodium nitroprusside, HbA1c, glycosylated haemoglobin.

However, this does not fully compensate for differences in starting conditions between groups and this is of particular relevance in diabetes as, in this condition, basal blood flow is influenced by the degree of glycaemic control (Elliott *et al.*, 1993).

The techniques of laser Doppler perfusion imaging and iontophoresis have been combined, enabling the reproducible measurement of skin blood flow responses to vasoactive agents (Morris *et al.*, 1995). Iontophoresis is a non-invasive method of drug application, which allows the local transfer of charged substances across the skin using a small electric current. A laser Doppler perfusion imager allows the measurement of skin erythrocyte flux directly over the site of drug application. Impaired responses of the skin microcirculation to iontophoretically applied ACh and SNP have been demonstrated in patients with both Type 1 and Type 2 diabetes (Morris *et al.*, 1995; Lim *et al.*, 1999; Katz *et al.*, 2001).

More recently, the development of a second non-invasive technique, the measurement of artery diameter and blood flow velocity, using high-resolution Doppler ultrasound has enabled further assessment of endothelial function of conduit vessels (mainly the brachial artery) in humans (Celermajer, 1998). The method has been used to evaluate the response to an increase in flow generated by a period of hyperaemia, which is considered to lead to flow mediated NO release (Sorenson *et al.*, 1995). The results from such studies are again contradictory with endothelium-dependent vascular responses shown to be impaired (Zenere *et al.*, 1995; Lekakis *et*

al., 1997; Morris *et al.*, 1995; Enderle *et al.*, 1998) or unchanged (Lambert *et al.*, 1996; Enderle *et al.*, 1998).

The conflicting data obtained in human *in vivo* studies could be attributed to a number of factors including methodological differences (e.g. method of study, agonists used) and variations in patient selection (e.g. sex differences, presence or absence of diabetic complications). The mechanisms whereby diabetes is associated with endothelial dysfunction are complex and not completely understood and are probably multiple and composed of both systemic and local factors. It has been demonstrated that patients with strict glycaemic control have a lower incidence of vascular complications (Diabetes Control and Complications Trial Group, 1993), thereby implicating hyperglycaemia as an independent risk factor for the development of cardiovascular disease. The mechanism by which elevated glucose contributes to the pathogenesis of vascular dysfunction is not completely understood but there is much evidence to suggest that the action of both glucose and insulin can directly alter the function of cells in the vascular wall (Hopfner & Gopalakrishnan, 1995; Porta *et al.*, 1987).

1.5.2.2. *In vitro* studies

Analysis of arteries isolated from patients with diabetes is complicated by the difficulties associated with obtaining suitable, viable tissue. Consequently, there have been relatively few *in vitro* studies of vascular function in diabetes using human vessels. Those investigations that have been performed have used sub-cutaneous resistance arteries taken from biopsies of the gluteal region (McNally *et al.*, 1995) or

lower extremities (Cipolla *et al.*, 1996). Resistance artery function can be studied using the technique of small vessel myography (Mulvany & Halpern, 1977) and has the advantage of using resistance vessels which have a major influence on the maintenance of peripheral resistance. These have produced contradictory results and, thus, have failed to confirm the influence of diabetes on either contraction or relaxation of resistance arteries. For example, both impaired (McNally *et al.*, 1995) and normal (Malik *et al.*, 1999) endothelium-dependent relaxation in response to ACh has been reported in arteries from Type 1 diabetic patients. The normal response observed in the latter study was despite an enhanced relaxation to an NO donor (SNP). Similarly, relaxation to ACh has also been reported to be normal, or even enhanced (Cipolla *et al.*, 1996) in vessels isolated from patients with Type 2 diabetes (Lawrence *et al.*, 1994). Intriguingly, the relaxation reported by McNally *et al.* (1995) was selective for ACh, with responses to bradykinin and SNP unaltered. This suggests a selective abnormality in the muscarinic receptors rather than a generalised endothelial cell dysfunction or impaired sensitivity to NO.

The effect of diabetes on contractile function is also unclear. One study reported a general impairment in contraction in arteries from patients with Type 1 diabetes (McNally *et al.*, 1995) whereas another found a selective enhancement in AII-mediated contraction (Malik *et al.*, 1999). In Type 2 diabetes, NA-mediated contraction of resistance arteries was enhanced as a result of an impairment in the ability of the endothelium to modulate the contractile response (Cipolla *et al.*, 1996). Intriguingly, ACh-mediated relaxation was not impaired in these vessels, suggesting a selective impairment in either basal or adrenoceptor-mediated NO release.

1.5.3. *Animal studies*

The difficulties encountered when studying vascular function in diabetic patients has prompted the development, and widespread use, of a number of animal models for research purpose. Diabetes can occur spontaneously in some animal species or can be induced by treating animals with chemicals or viruses. The most commonly used animal models are the alloxan-induced diabetic rabbit, the streptozotocin (STZ)-induced diabetic rat and the genetically susceptible bio-breeding (BB) rat (Bell & Hye, 1983). Both alloxan and STZ cause selective ablation of the pancreatic β -cells, although sufficient β -cell activity remains which obviates the need for exogenous insulin. These animals exhibit hyperglycaemia and other characteristics of diabetes such as polydipsia, polyphagia and polyuria (Tomlinson *et al.*, 1992). The BB rat is a model of spontaneous diabetes that exhibits a total lack of insulin secretion from pancreatic β -cells. BB rats are hyperglycaemic, hypoinsulinaemic, have significant polyuria and glycosuria, and need insulin administration to survive (Nakhooa *et al.*, 1977).

Most studies of vascular function in diabetic animals have focused upon determination of endothelium-dependent relaxation. In isolated arteries prepared for isometric force measurement, endothelium-dependent relaxation is assessed by pre-contraction of the artery with a suitable agonist (usually NA) and by the subsequent determination of relaxation to an endothelium-dependent vasodilator (usually ACh). As described earlier, (Chapter 1.2 & 1.3), ACh-induced vasodilatation is mediated by release of NO (Moncada *et al.*, 1991), PGI₂ (Woolfson & Poston, 1990), or EDHF (Garland & McPherson, 1992). The relative contribution of these dilating factors

varies in arteries from different territories. It is unlikely that diabetes would produce the same pathophysiological changes in conduit and resistance arteries, as it is well established that these vessels are functionally distinct. Receptor populations vary in arteries of different anatomical origin (Cambridge *et al.*, 1995) and, perhaps more significantly, the factors that contribute to endothelium-dependent relaxation differ in large compared with small vessels. Endothelium-dependent relaxation is mediated almost exclusively by NO in conduit arteries (Vanhoutte, 1989), whereas the contribution of EDHF becomes increasingly significant as the diameter of the vessel diminishes (Shimokawa *et al.*, 1996). Most studies in conduit arteries from diabetic animals have shown an attenuation of the normal vasodilatory response to ACh (Poston & Taylor, 1995). The defect appears to lie at the level of the receptor rather than an inability of the endothelial cells to release, or of the vascular smooth muscle cells to relax to NO, as responses to Ca^{2+} ionophores and NO donors remain normal.

As with human *in vitro* studies, investigation of resistance artery function using vessels from animal models has failed to determine the exact nature of vascular dysfunction in diabetes. In resistance arteries from diabetic rats, endothelium-dependent relaxation has been found to be impaired, normal or even enhanced (Chan *et al.*, 2000). This disparity may be due to differences in disease duration, the vascular bed studied and the methods of study (perfusion versus isolated tissue preparation). The importance of disease duration has been highlighted in a recent study that demonstrated a triphasic response in relation to disease duration in the STZ-induced diabetic rat (Pieper, 1999). ACh-induced endothelium-dependent

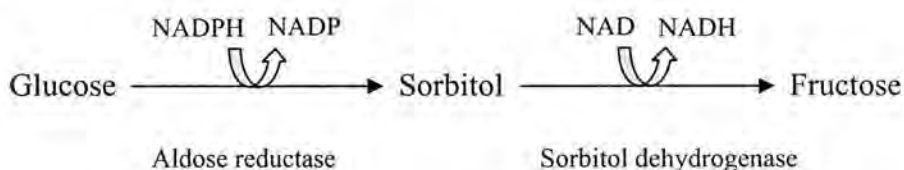
relaxation was increased 24 hours following disease induction, normal after 1-2 weeks and impaired after 8 weeks of the disease.

1.6. Development of diabetic microvascular complications: the role of hyperglycaemia.

The profound association of elevated blood glucose with the development of micro- and macrovascular complications of diabetes suggests strongly that hyperglycaemia may directly alter vascular physiology. Multiple mechanisms have been suggested for the possible pathway(s) by which hyperglycaemia could cause vascular dysfunction. These include a role for aldose reductase and the polyol pathway, free radicals, protein kinase C and advanced glycosylation end products. Each of these pathways implicated in hyperglycaemia-induced endothelial dysfunction will now be discussed.

1.6.1. Aldose reductase and the polyol pathway

Hyperglycaemia leads to an increase in glucose metabolism through the polyol pathway (Hawthorne *et al.*, 1989), which increase sorbitol flux and fructose synthesis through enhanced aldose reductase activity.



Aldose reductase, the rate-limiting enzyme in the conversion of glucose to sorbitol, is present in high amounts in endothelial cells (Kern & Engerman, 1982). The activity

of aldose reductase requires and may deplete NADPH, which is an essential co-factor for the generation of NO from arginine by the enzyme NOS. Hence, NADPH depletion as a result of chronic hyperglycaemia could lead to a reduction in NO production. The importance of increased polyol formation in diabetic endothelial dysfunction is further supported by the observation that aldose reductase inhibitors restore endothelium-dependent relaxations in diabetic animals and reverse the effect of hyperglycemia-induced endothelial cell dysfunction (Cameron & Cotter, 1992; Williamson *et al.*, 1990; Tesfamariam *et al.*, 1993).

1.6.2. Vasoconstrictor Prostanoids

In aortae from diabetic rabbits and normal rabbits exposed to elevated glucose *in vitro*, impaired relaxations to acetylcholine are restored by treatment with various cyclooxygenase inhibitors (Tesfamariam *et al.*, 1989; Tesfamariam *et al.*, 1990), indicating that contractile prostanoids could counteract NO-mediated relaxations. The potent vasoconstrictor prostanoid, thromboxane A₂, is unlikely to account for the impaired relaxation as a TxA₂ synthase inhibitor failed to prevent the abnormal relaxations of aortae exposed to elevated glucose (Tesfamariam *et al.*, 1990). This leaves open the possibility that other prostanoids are important. PGH₂/TxA₂-receptor blockade also prevents the abnormal endothelium-dependent relaxations of diabetic aortae and aortae exposed to elevated glucose, suggesting that PGH₂, the precursor prostaglandin, or other prostaglandins could account for the impairment.

1.6.3. *Free radicals*

The formation of oxidation products that are potentially damaging to the endothelium depends upon the balance between pro- and antioxidant status. There is abundant evidence in diabetes for an increase in oxidative stress, where pro-oxidants outweigh antioxidants (reviewed Wolff, 1993). This increased stress may be due to elevated free radical generation, decreased levels of antioxidants and/or impaired regeneration of reduced forms of antioxidants. An increase in free radical synthesis may occur through the autoxidation of glucose (Wolff, 1993) or through a glucose-induced increase in activity of the cyclooxygenase pathway (Tesfamariam & Cohen, 1992).

There is substantial evidence to indicate that hyperglycaemia-induced endothelial cell dysfunction is mediated by free radicals produced through increased arachidonic acid metabolism (Tesfamariam & Cohen, 1992; Tesfamariam, 1994; Giugliano *et al.*, 1996). In addition it has been demonstrated that in human aortic endothelial cells glucose-induced increases in eNOS expression and NO release is associated with a concomitant increase in superoxide anion generation (Cosentino *et al.*, 1997). Superoxide anions inactivate NO and furthermore, interact with NO to produce peroxynitrite (Squadrito & Pryor, 1995), which results in endothelial dysfunction through increased cyclooxygenase catalysis, lipid peroxidation and increased prostanoid production (Tate *et al.*, 1984; Jackson *et al.*, 1986). A role for superoxide anions in glucose-induced endothelial-dysfunction is further demonstrated by the fact that superoxide dismutase, a superoxide anion scavenger, normalises glucose-impaired NO-induced relaxation (Hattori *et al.*, 1991; Diederich *et al.*, 1994).

1.6.4. *Protein kinase C*

Activation of PKC by increases in DAG induced by hyperglycaemia has been suggested as a mechanism for endothelial dysfunction and vascular complications in diabetes (Wolf *et al.*, 1991). Normal blood vessels treated for just a few minutes with phorbol esters, which activate PKC, develop endothelium-dependent relaxations that are impaired like those of diabetic blood vessels, and the abnormal relaxation can be attributed in part to the increased production of vasoconstrictor prostanoids (Tesfamariam *et al.*, 1991). Furthermore, the abnormal endothelium-dependent relaxation was restored with sphingosine, a PKC inhibitor. The PKC inhibitor also prevented the glucose-induced increase in prostanoid production, suggesting that glucose impairs endothelium-dependent relaxation by a PKC-mediated production of prostanoids.

1.6.5. *Advanced glycosylation end products*

When plasma and cell membrane proteins are exposed to high concentrations of glucose for prolonged periods, they undergo nonenzymatic glycosylation, cross-linking, and formation of advanced glycosylation end products (AGEs, Brownlee *et al.*, 1988). The resulting altered proteins may cause cellular dysfunction and have been implicated in the complications of diabetes. AGEs have been shown to inactivate NO *in vitro* and aminoguanidine, an inhibitor of AGEs formation, has been shown to partially restore endothelium-dependent relaxations of diabetic rat aortae (Bucala *et al.*, 1991), suggesting that advanced glycosylation end products may be responsible for endothelial dysfunction in chronic diabetes. AGEs form slowly over days to weeks *in vitro* (Brownlee *et al.*, 1988), and are therefore unlikely to play a

role in the effect of elevated glucose applied to blood vessels *in vitro*. It should also be noted that aminoguanidine is a structural analogue of arginine that actually inhibits NOS (Corbet *et al.*, 1992) and therefore could have a direct effect on NO generation.

1.7. Hypothesis and Aims

The work carried out in this thesis studied the hypothesis that the vascular abnormalities present in the microvasculature in Type 1 and Type 2 diabetes are the consequence of exposure to elevated blood glucose concentrations.

To date few investigators have examined vascular function *in vitro* in human arteries isolated from patients with Type 1 and Type 2 diabetes. Therefore the aims of this thesis were threefold:

- (1) to refine a protocol that could be used for isolating and identifying resistance arteries isolated from patients with diabetes
- (2) to determine whether endothelial and/or vascular smooth muscle function are altered in diabetes and
- (3) to examine the effects of acute elevations of glucose on resistance artery function

CHAPTER 2

METHODS

2.1 SMALL VESSEL MYOGRAPHY

2.1.1. *Introduction.*

Prior to the mid 1970s, investigations into the structure and function of isolated vessels had been confined to the aorta and arteries with lumen diameters greater than 500 μ m. However, it was recognised that it is the smaller arterial vessels (100-500 μ m) which present the greatest resistance, and which are most involved in regulating blood flow and capillary pressure (Furness & Marshall, 1974). Until the 1970's, characteristics of resistance vessels could only be inferred, primarily from haemodynamic studies, or by extrapolation from characteristics of elastic and conduit arteries.

A major technical breakthrough (Bevan & Osher, 1972) made possible the study of isolated resistance arteries *in vitro*. This technique, small vessel myography, allowed direct mechanical measurements on intact segments from small (less than 500 μ m) vessels by mounting them as ring preparations horizontally on two fine wires, with the wires clamped at each end to ensure that the responses were isometric. In addition, this technique allowed measurement of media: lumen ratio under standard conditions and enabled analysis of function and structure in the same vessel (Mulvany & Halpern, 1976). Further refinement allowed investigation of vessels with internal diameters as small as ~100 μ m (Mulvany & Halpern, 1976, 1977; Mulvany *et al.*, 1978), a dual myograph for simultaneous testing of two vessels was introduced by Mulvany & Nyborg (1980), and isobaric (pressure) myographs have also been developed (Duling *et al.*, 1981).

Myography is relatively atraumatic, with the measured responses exceeding the estimated *in vivo* responses and corresponding to the force-generating capacity known from other smooth muscle preparations (Mulvany & Halpern, 1977). The presence of intraluminal wires does cause some damage to the endothelial cell layer, but does not impair endothelium-dependent relaxation (Falloon *et al.*, 1995). The main criticism of wire myography is that it is not physiological: the vessel rings are flat, rather than cylindrical and are not exposed to shear forces. This is reflected in the demonstration that vessel sections in a wire myograph are less depolarised than those in perfusion systems and, as a consequence, develop less myogenic tone and are less sensitive to vasoconstrictors (Buus *et al.*, 1994; Dunn *et al.*, 1994). Despite these imperfections, wire myography provides a valuable tool for investigations of vascular physiology and pathophysiology. It allows the determination of the following parameters for vessels with diameters in the range 100-1000 μ m:

- (a) normalised internal diameter (the lumen diameter under a known stretch).
- (b) Thickness of the adventitial, medial and intimal layers of the vascular wall.
- (c) isometric response to agonists.
- (d) simultaneous measurements of isometric response and membrane potential.
- (e) simultaneous measurements of isometric response and cytoplasmic ion concentration.
- (f) fixation of vessels under clearly defined conditions for histometric analysis.

The small vessel myograph used in the experimental procedures during this thesis was the dual myograph model 410A, manufactured by J.P. Trading, Science Park

Aarhus, Aarhus C, Denmark. Functional analyses were developed on the basis of information supplied in the course on Basic Myography run by Professor Mulvany at the University of Aarhus.

2.1.2. *Isolation of small resistance arteries*

2.1.2.1. Rat mesenteric vessels.

Male Cob-Wistar rats, maintained on standard chow and tap water *ad libitum*, were obtained from the Animal Unit, Department of Pharmacology, University of Edinburgh. The animals (150-250g) were killed by stunning followed by cervical dislocation and a ventral mid-line incision was made. The mesenteric bed was removed and immediately placed in physiological salt solution (PSS) of the following composition (mM): - NaCl 119, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.17, NaHCO₃ 25, KH₂PO₄ 1.18, K₂EDTA 0.026 and D-glucose 5.5. The mesenteric bed was then pinned out in a silicone coated (Sylgard, Dow-Corning, UK) petri dish containing PSS and dissection was performed at room temperature.

For resistance arteries, approximately 200-300µm in diameter, third order branches of the superior mesenteric artery, were excised under a dissection microscope (x20 magnification) (Zeiss, UK) using No. 5 watchmaker forceps (Merck, Poole, Dorset, UK) and fine trabecular dissection scissors (Geuder G-19745, Altomed Ltd, Tyne & Wear, UK). To avoid direct contact with the section of vessel to be studied, surrounding connective tissue was gently pulled away from the artery to expose the thin membrane connecting the connective tissue to the artery. This membrane was

cut, thus isolating the artery from the fat cells. The artery, along with the feeder artery, was then excised.

2.1.2.2. Human vessels: tissues from gluteal fat biopsies.

Skin biopsies, approximately 2cm long, 0.75cm wide and 0.75cm deep, were taken from the gluteal region under local anaesthesia (2% Lignocaine hydrochloride, Astra Pharmaceuticals Ltd, UK) (Aalkjaer *et al.*, 1986). The biopsies were immediately immersed in cold PSS and brought to the laboratory. Arteries (usually 1-3) were dissected out from each biopsy using light microscopy. In order to distinguish arteries from veins the following guidelines were followed:

1. the artery is stiffer than the vein, and sometimes sticks out of the biopsy.
2. the walls of arteries are thicker and more distinct than walls of veins.
3. if you pull the connected tissue the vein will flatten out, while the artery tends to keep its shape.
4. the artery is often smaller than the vein and when dissected from the biopsy the artery retains its shape whilst the vein tends to curl up.

2.1.3. *Mounting and normalisation of vessels in the myograph*

2.1.3.1. Mounting of the vessel in the myograph.

Once dissected, the arteries were carefully transferred to the myograph chamber, which contained 12.5ml of PSS, for mounting on 40µm wires. The first wire was positioned in the centre between the jaws and the jaws were then screwed together so that the wire was clamped. The far end of the wire was bent towards the movable jaw and wrapped around under the fixing screw in a clockwise direction so that tightening the screw also tightened the wire. This resulted in the wire being clamped

between the jaws with the near end pointing towards the operator. Using the feeder artery, the vessel was slid onto the end of the wire (if the lumen was not patent, the wire tip was used to gently open the end of the vessel). Once the vessel was on the wire the jaws were opened to free the wire. The proximal end of the vessel was then gently pulled along the wire with care being taken not to stretch the vessel if the end of the wire caught on the vessel wall. The vessel was pulled along the wire until the segment to be investigated was situated between the mounting jaws. Once in position, the jaws were screwed together to clamp the wire, ensuring that the bottom end of the vessel was inside the jaw gap, such that it was not touched by the jaws. The near end of the wire was then secured under the near fixing screw on the movable jaw, again in a clockwise direction, so that tightening the screw also tightened the wire. The 'feeder' segment of the artery on the far side of the jaw was gently rubbed with forceps and after about half a minute this severed the vessel so that the portion of the 'feeder' artery on the far side of the jaw could be discarded. The jaws were then separated to allow insertion of the second wire through the vessel lumen. The wire was then gently fed through the lumen, with care taken to ensure that the end of the wire did not catch on the vessel wall. When the second wire was fully passed through the vessel it was positioned with the vessel at the centre and the jaws were screwed back together, ensuring that it moved under the first. The second wire was then secured around the fixing screws of the fixed jaw. With both wires secured, the jaws were separated so that the vessel was slightly stretched and final adjustments were made to ensure that the wires were level (Figure 2.1).

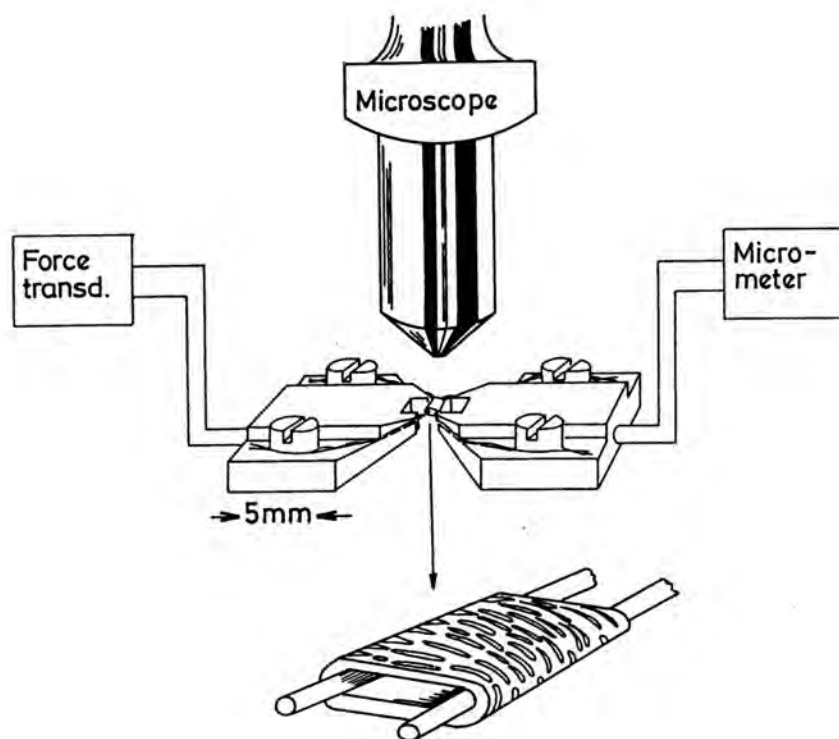


Figure 2.1. Schematic diagram of the wire myograph, where vessel segments are threaded on two 40 μ m stainless steel wires that are fastened to a micrometer and a force transducer, for measurement of isometric tension. (Figure taken from Mulvany & Aalkjaer, 1990).

In order to measure the vessel length, a travelling micrometer eyepiece was attached to the microscope and the microscope was set to the magnification at which the eyepiece was calibrated (x40). Measurement of the vessel length was achieved using the travelling micrometer. Once the arteries were mounted, the PSS in the myograph was replaced, perfused with 95% O₂ and 5% CO₂ and equilibrated at 37°C.

2.1.3.2. Determination of normalised lumen using small vessel myograph

Following a 30min equilibration period, to allow the PSS in the myograph chamber to reach 37°C, the vessel was then normalised. This procedure determines for a vessel mounted on the myograph the internal circumference, denoted IC₁₀₀, which the vessel would have if relaxed and under a transmural pressure of 100mmHg. This procedure is necessary as the sensitivity of preparations to agonists is dependent on the degree of stretch (Nilsson & Sjöblom, 1985; Mulvany 1983).

Normalisation was performed by stretching the vessel in steps and recording micrometer and force (measured on a dual channel flat bed pen recorder (Sekonic SS-250F)) readings for each stretch. Each pair of readings was keyed into a computer program ("Normalisation" program, gift from Dr. N. Stevens, Manchester University; adapted to Windows format by Mr. D. Christie, Dept. Medicine, University of Edinburgh) and, having entered the transducer calibration, the effective pressure corresponding to each stretch was calculated. This procedure was completed once the effective pressure exceeded 100mmHg (13.3kPa). The program fits an exponential curve to the data and, using LaPlace's equation, the point on the curve corresponding to 100mmHg is determined and denoted IC₁₀₀. Having found this, the

stretch on the vessel was adjusted to give an internal circumference of $0.9IC_{100}$, which has been shown to be the optimum resting setting for these vessels (Mulvany & Halpern, 1977).

2.1.4. Assessment of vessel viability

In order to confirm the viability of each vessel section and the reproducibility of contraction, vessels were subjected to a standard start procedure (Aalkjaer *et al.*, 1987).

The standard start was performed after the vessel had been equilibrated at its normalised setting at 37°C. It consisted of a series of five stimulations of 3 minutes each followed by washout periods of 5 minutes in PSS to allow full relaxation. The first, second and fifth contractions were produced using NA-K, a high potassium solution (125mM KPSS, made by the equimolar substitution of KCl for NaCl in PSS) containing 10µM noradrenaline (NA). The third contraction was produced by KPSS alone and the fourth by NA (10µM). This procedure confirms the reproducibility of contraction and demonstrates which component is due to K^+ and which is due to NA (important e.g. in mouse aorta). According to convention (based upon the active force produced by smooth muscle cells (Mulvany & Halpern, 1976)) a viable resistance artery should produce an effective pressure in excess of 13.3kPa in response to the final exposure to NA-K.

2.1.5. *De-endothelialisation.*

The purpose of de-endothelialisation is to eliminate endothelial effects on the rest of the vessel (e.g. to examine smooth muscle function in the absence of endothelium-derived factors). The endothelium removal can be used either to rule out the influence of the endothelium on a certain mechanism, or to confirm the role of the endothelium in the mechanism (this can only be done if endothelium intact vessels are compared with denuded vessels). In large arteries the most common approach for removing the endothelium is by mechanical means, usually by rubbing the inner surface of a vessel segment with a small stick or wire (Osol *et al.*, 1989). This technique is difficult to apply to small resistance arteries because of their fragility and therefore a wide range of alternative non-mechanical methods have been tried; for example, perfusion with a dilute detergent (DeMay & Gray, 1985), dissolving the intracellular matrix with enzymes such as collagenase or elastase (Harder, 1987), or rupturing endothelial cells osmotically with distilled water (Bolton *et al.*, 1984). Unfortunately, these chemical and enzymatic methods are difficult to control precisely (e.g. exposure time, shear rate) and carry the risk of damaging adjacent smooth muscle cells. It then becomes difficult to know with certainty whether an observed difference in the vessel response is due specifically to endothelial cell removal. Alternative ways of denuding resistance arteries mechanically are the introduction of a single human hair into the vessel lumen (Osol *et al.*, 1989) and perfusion of the lumen with an air bubble (Falloon *et al.*, 1993). The use of a human hair provides a simple and effective tool for mechanical denudation of small arteries as the surface of the hair is uneven and consists of repeating ridges or scales with jagged edges which provides a reasonable degree of cellular abrasion. Both

functional and morphological evidence have demonstrated that this technique for denudation is effective, and that smooth muscle cell damage can be either absent, or very minimal (Osol *et al.*, 1989; Gustafsson & Nilsson, 1990), although this is obviously operator dependent.

2.1.5.1. Removal of endothelium using a human hair.

Removal of the endothelium was achieved by passing a human hair through the lumen of the mounted artery, according to the method described by Gustafsson & Nilsson, (1990). Following normalisation and the standard start procedure to ensure that the vessel was viable, the functional integrity of the endothelium was checked by adding acetylcholine (ACh; 10 μ M) to vessels submaximally contracted with NA (3 μ M). Following this, the vessel was set to a small tension (0.5mN) and under a microscope magnification of x40 the tip of the hair was inserted into the vessel lumen. The hair was then worked down through the whole inner surface of the vessel rubbing carefully and cautiously forwards and backwards. Care was taken to avoid pushing the tip into the vessel wall and thereby damaging the muscle layer. It was also necessary to avoid stretching/compressing the vessel in its longitudinal direction when the hair was moved forward/backwards, as this damages the vessel and causes a significant impairment in contraction. After the procedure, the solution in the chamber was changed and the vessel was allowed to equilibrate for 5-10min. Following equilibration, the vessel was slowly stretched back to its normalised micrometer setting and was allowed to relax for 5min before repeating the test for endothelium-dependent relaxation. A successful endothelium-removal was classed as abolition of ACh-mediated relaxation but with minimal reduction of active tension.

In general, it was not possible to perform this procedure without some reduction in contractility and often a small amount of residual endothelium-dependent relaxation remained.

2.1.6. *Functional Analysis.*

2.1.6.1. Vasoconstriction

The use of vasoconstrictors acting via different mechanisms should indicate whether changes in contraction are due to a loss of receptor function or a general inability of the smooth muscle cells to contract. For this reason three vasoconstrictors were chosen, KCl, NA and endothelin-1 (ET-1). KCl was selected as it causes smooth muscle contraction by direct depolarization, whereas NA and ET-1 were selected as examples of agonists which produce receptor-mediated constriction. A further use of the concentration-responses curve to NA was that it determined which concentration produced a suitable contraction for investigation of vasodilator compounds.

2.1.6.2. Vasodilatation

It would appear that the most important mechanism of vasodilatation altered by diabetes is the endothelium-dependent nitric oxide (EDNO) system. Therefore, it would be necessary to determine whether any alteration in relaxation was due to an impaired release of endothelium-derived relaxing factors or a general inability of the vascular smooth muscle cells to relax. For this reason four vasodilators were chosen: ACh, bradykinin (BK), A23187 and 3'-morpholinonydnonimine (SIN-1). Both ACh and BK are receptor-dependent, endothelium-dependent vasodilators, but act on different receptors. The Ca^{2+} ionophore A23187 is a receptor-independent,

endothelium-dependent agonist. The employment of SIN-1, an endothelium-independent vasodilator, is necessary to ensure that the vessel retains the ability to relax.

2.1.7. *Statistics*

The influence of manipulations *in vitro*, or the presence of diabetes *in vivo*, on functional responses were determined using summary statistics. Maximum response (Emax) and sensitivity (EC₅₀) values were obtained from concentration-response curves using Fig P (Biosoft, UK). Data were analysed using Graph Pad (USA) software (Statmate, Instat) to assess statistical power, normal distribution and equality of variance. Comparisons were made using a variety of statistical tests: Student's paired/ unpaired t-test, Mann-Whitney test, one-way analysis of variance (ANOVA) followed by Dunnet's or Tukey post-hoc tests or Kruskall-Wallis followed by Dunn's post hoc test, as appropriate.

2.1.8. *Drugs.*

Stock solutions were prepared for each drug and these were stored in 1ml aliquots at -20°C. Drugs were thawed as required, subsequent dilutions were made in distilled water and any residual solution was discarded at the end of the experiment. A list of the drugs used throughout the course of this thesis is shown in Table 2.1. All salts were supplied by BDH (Poole, Dorset, U.K) and Insulin (human Velosulin 100U/ml) was obtained from NovoNordisk (Crawley, West Sussex, U.K.).

Table 2.1. A list of the drugs used throughout the course of this thesis.

DRUG	STOCK	SUPPLIER
<i>Vasoconstrictors</i>		
Noradrenaline	10^{-3} M in dH ₂ O	Sigma
endothelin-1	10^{-5} M in 50% MeOH	Alexis
potassium chloride	125mM *	BDH-Merck
<i>Vasodilators</i>		
acetylcholine	10^{-3} M in dH ₂ O	Sigma
bradykinin	10^{-4} M in dH ₂ O	Sigma
A23187	10^{-4} M in 10% EtOH	Sigma
SIN-1*	10^{-3} M in dH ₂ O	Alexis

dH₂O, distilled water; EtOH, ethanol; MeOH, methanol; SIN-1, 3'-morpholinosydnonimine.

*SIN-1 was also obtained as a gift from Dr. K. Schonafinger, Cassella, Germany.

125mM KCl made by the equimolar substitution of KCl for NaCl in PSS.

ALEXIS CORPORATION (UK) LTD, 3 Moorbridge Court, Moorbridge Road East, Bingham, Nottingham, England, UK.

BDH LABORATORY SUPPLIES, Poole, Dorset, England, UK.

SIGMA-ALDRICH COMPANY LTD, Fancy Road, Poole, Dorset, England, UK.

2.2. MORPHOLOGICAL ANALYSIS.

Since limited information is available regarding the structure of human subcutaneous resistance arteries, histological staining was used to complement the results obtained from the functional investigations.

Immunohistochemistry has become an established routine histological technique for the identification of tissue constituents (antigens). The basis of this methodology relies on antigen-antibody interactions, with the site of antibody binding being identified either by direct labelling of the Ab, or by use of a secondary labelling method. Hence, this means that this technique can be used to detect anything which is antigenic in nature, producing highly specific results.

2.2.1. *Fixation of vessels.*

Human subcutaneous resistance arteries were isolated as previously described, and immediately fixed in 10% neutral buffered formalin solution for 24hrs at room temperature. In addition to freshly isolated vessels, those which had previously been used for functional investigations were carefully removed from the wires at the end of the experiment and also fixed. Following fixation, the tissues underwent a routine processing schedule (Pathology Department, University of Edinburgh) and were embedded in paraffin wax (60°C melting point). Due to the inherent fragility and size of the microvessels, embedding in paraffin wax using normal procedures proved difficult as the vessel was difficult to locate in the wax and proper orientation of the vessel was not always achieved. In order to overcome these obstacles the tissues were wrapped in paper before being embedded in the wax. In this way the vessels

could be manipulated using the paper, thus avoiding any damage to the vessel, and embedding in the wax in the proper orientation was easily achieved. In order to identify the very small pieces of tissue at the embedding stage a few drops of eosin was added before transferring the tissue to the cassette. The pink coloration of the tissues remains during processing but is washed out during section staining.

Once embedded in the wax, serial 3µm sections were cut using a rotary microtome (Leitz 1515), mounted from water at 50°C onto poly-L-lysine coated glass slides (BDH-Merck, Poole, Dorset, U.K.), to avoid the sections floating off during staining, and dried overnight in an oven at 37°C.

2.2.2. *Haematoxylin and eosin*

General morphology was established using the classical histochemical combination of haematoxylin and eosin. Haematoxylin is a basic dye which stains cell nuclei blue/black, whilst eosin is a red anionic dye which stains cell cytoplasm and most connective tissue fibres in various shades of red.

The tissue sections were deparaffinised in xylene for 5 minutes twice and rehydrated through a descending alcohol series (100%-90%-70% ethanol) for 3 minutes each, followed by thorough washing in tap water for 5 minutes. Harris haematoxylin was added for 30 seconds followed by washing in tap water for 3min. The sections were then treated with eosin (1% in tap water) for 2-3 minutes after which the nuclear staining was checked microscopically. The sections were then rinsed in water,

rapidly dehydrated through an ascending alcohol series, cleared in xylene and coverslipped using DPx mountant (BDH-Merck).

2.2.3. Immunohistochemistry.

Immunohistochemistry is a technique used for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions. Immunohistological staining was performed using a three-step indirect avidin-biotin-peroxidase complex (ABC) method. In this technique the unconjugated primary antibody (1° Ab) is applied, followed by a biotinylated labelled secondary antibody (2° Ab) directed against the 1° Ab. This is then followed by the ABC conjugated to peroxidase which, in combination with the chromagen 3,3'-diaminobenzidine tetrahydrochloride (DAB), yields an insoluble, stable, dark brown reaction end product (Figure 2.2).

2.2.3.1. Immunohistochemical Protocol

Tissue sections were dewaxed in xylene for 5 minutes (twice) and rehydrated through a descending alcohol series (100%-90%-70% ethanol) for 3 minutes each followed by a thorough washing in tap water. Endogenous peroxidase activity was inhibited by treating the sections with freshly prepared methanolic H₂O₂ (1 part 3% H₂O₂ in 4 parts absolute methanol) for 30 minutes, followed by washing in distilled water (Streefkerk, 1972).

In order to unblock any antigenic sites that may have been masked during fixation the sections were treated with the proteolytic enzyme trypsin. All sections were

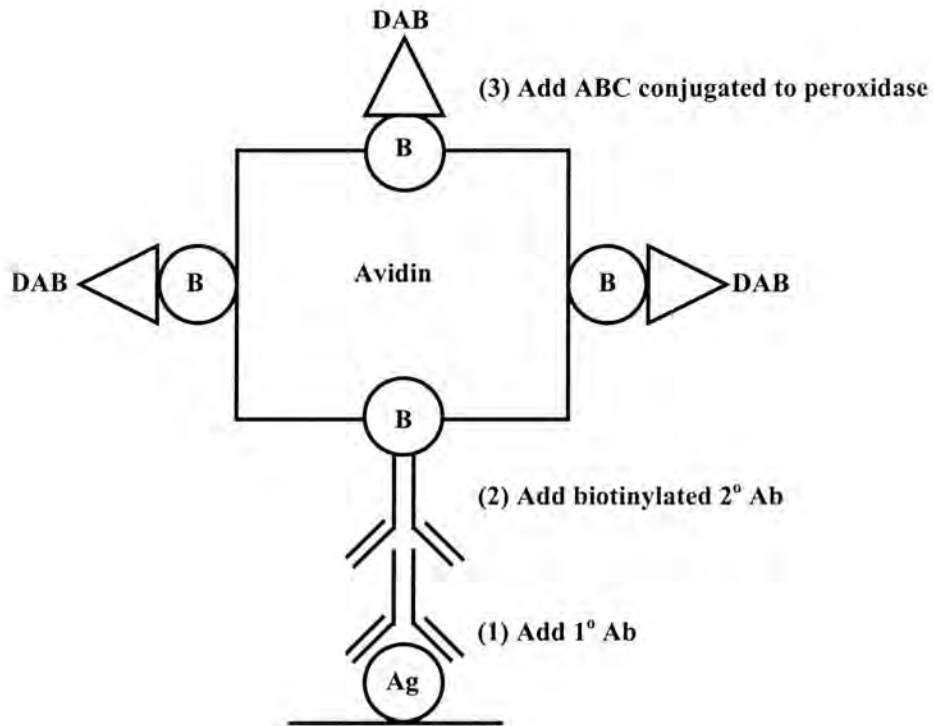


Figure 2.2. Schematic diagram showing the three-step indirect avidin biotinylated enzyme complex (ABC) immunohistochemical technique. The primary antibody (1° Ab), directed against the antigen (Ag), is incubated overnight at 4°C. A secondary biotinylated antibody (2° Ab) is added, followed by addition of the preformed ABC conjugated to peroxidase (Δ). To visualise the sites of positive binding, the chromagen 3,3-diaminobenzidine tetrahydrochloride (DAB), which is the substrate for peroxidase, is added, producing an insoluble brown precipitate.

preheated to 37°C in a coplin jar filled with distilled water before transferring to a coplin jar containing prewarmed 0.05M Tris buffered solution (TBS) at 37°C for 10 minutes. The sections were then washed for 30 minutes in freshly prepared TBS containing 0.1% trypsin and 0.1% CaCl₂ (pH 7.8), preheated to 37°C. After washing in phosphate buffer solution (PBS) for 20 minutes, the sections were ringed and isolated on the slide by drawing round the tissues with a paraffin-wax pen (Dako Ltd, Buckinghamshire). In order to eliminate non-specific background staining, which may occur as a result of non-immunological binding of the 1° Ab by hydrophobic interactions to certain sites within the tissues sections, a solution of 1% normal goat serum was applied for 30 minutes at room temperature.

Following blocking, the primary antibodies, optimally diluted in 1% normal goat serum in PBS, were applied to sections and left overnight at 4°C (a list of the antibodies used is shown in Table 2.2). In order to test the specificity of the antibodies involved it is necessary to include controls. In this study, a negative control, which involves omission of the 1° Ab from the staining schedule, was included in the protocol.

Following overnight incubation with the primary antibody, the sections were brought to room temperature (30min) and washed in PBS for 20 minutes. The appropriate biotinylated secondary antibody (goat anti-mouse IgG, Dako Ltd, UK), diluted 1/200 in BSA/PBS, was applied for 30min at room temperature, where necessary. Following washing in PBS for 20 minutes, the ABC complex (freshly prepared 30 minutes before use) was applied to the sections for 30min at room temperature. After

Table 2.2. A list of the antibodies used for immunohistochemical staining of the endothelium, vascular smooth muscle and localisation of ET-1.

Primary Antibody	Working Dilution	Supplier
mouse anti ET-1 IgG	1/500	Ams Biotechnology
mouse anti smooth muscle actin, IgG	1/50	Novacastra Labs Ltd
biotinylated ulex europaeus agglutinin*	1/100	Vector Labs

All antibodies were optimally diluted in 1% BSA in PBS. *This antibody was already biotinylated and therefore did not require addition of the secondary antibody.

Ams Biotechnology, 5 Thorney Leys Park, Witney, Oxon.

Novacastra Labs Ltd, 24 Claremont Place, Newcastle Upon Tyne, NE2 4AA.

Vector Laboratories Ltd, 16 Wulfic Square, Bretton, Peterborough, PE3 8RF.

washing in PBS for 20 minutes, the DAB substrate was applied for 5 min in order to visualise sites of positive reactivity. The slides were rinsed in PBS and washed in running tap water for 5min before counterstaining the nuclei with haematoxylin, dehydrating through alcohols (70%-90%-100%), clearing in xylene and mounting in DPx.

2.2.4. Solutions.

2.2.4.1. Haematoxylin and eosin

Harris haematoxylin (Acidified) and eosin Y were obtained ready to use from Themoshandon (Runcorn, Cheshire, UK).

2.2.4.2. Immunohistochemistry.

(1) Phosphate Buffer Solution (PBS) used for washing

- (a) 12.7g disodium hydrogen orthophosphate was dissolved in 100ml distilled water by microwaving on full power for 5 minutes
- (b) 1.7g sodium dihydrogen orthophosphate was dissolved in 100ml distilled water
- (c) The two solutions were mixed and the volume made up to 1 litre with distilled water
- (d) The pH was adjusted to 7.6 using 4N HCl

(2) 0.05M Tris buffer for trypsinisation

- (a) 6.04g Trisma base dissolved in 80ml distilled water
- (b) 2.77ml 4N HCl was added to Tris solution
- (c) 8.1g NaCl was dissolved in 900ml distilled water

- (d) the 2 solutions were mixed, pH adjusted to 7.8 with 4N HCl, and the volume made up to 1 litre

(3) *Vector ABC complex kit*

- (a) to 5mls PBS, 2 drops of solution A and 2 drops of solution B were added
- (b) the solution was left to stand for 30min before use

(4) *DAB substrate*

to 1 aliquot (0.5ml) of DAB the following were added:-

- (a) 1.5ml Tris buffer with thymol
- (b) 60µl imidazole solution (0.045g imidazole in 1ml distilled water)
- (c) 30µl 1% H₂O₂
- (d) the solution was freshly prepared immediately before use

CHAPTER 3

EFFECT OF PROLONGED COLD STORAGE

ON RAT MESENTERIC ARTERIAL

REACTIVITY

3.1. Introduction

Isolated blood vessel segments are used extensively to investigate *in vitro* the physiological and pharmacological control of the vasculature and the mechanisms responsible for deranged vascular function in disease states. One of the major limitations of this type of investigation is the perceived need to use freshly isolated blood vessels. This is mainly due to the well-documented fragility of the vascular endothelium, which may be damaged both during isolation and storage (Kristek *et al.*, 1993; Torok *et al.*, 1993), in contrast to smooth muscle which can remain viable for up to eight days when stored at 2-6°C (Kristek *et al.*, 1993). The requirement that blood vessels must be freshly isolated restricts the number of investigations which can be performed on vessel segments from a single animal and is even more problematic when studying human blood vessels, since the availability of such vessels can be both irregular and unpredictable. Consequently, a straightforward and reliable method for storing vessels prior to experimentation is desirable and this idea has stimulated investigations into both refrigeration (2-6°C; Shibata, 1969; Carrier *et al.*, 1973; Kristek *et al.*, 1993; Torok *et al.*, 1993) and low temperature (-70 or -190°C) cryopreservation of blood vessel samples (Muller-Schwienitzer *et al.*, 1986; Ku *et al.*, 1990).

Most studies carried out to date on the effects of storage of blood vessels on vascular reactivity have been performed using large conduit vessels (Shibata, 1969; Carrier *et al.*, 1973; Kristek *et al.*, 1993; Torok *et al.*, 1993) rather than small vessels. Following the introduction of small vessel myography (Mulvany & Halpern, 1977),

however, it has become desirable to preserve vascular function in small resistance arteries for a prolonged time period.

3.2. Aim

The aim of the experiments described in this study was, therefore, to determine whether vascular smooth muscle and endothelial cell function could be preserved in resistance arteries by storing them in physiological salt solution (PSS) at 4°C.

3.3. Methods

3.3.1. *Experimental set-up*

Male Cob-Wistar rats (150-250g) were killed by stunning followed by cervical dislocation. The mesenteric bed was removed and placed in cold (4°C) PSS of the following composition (mM) :- NaCl, 119; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.17; NaHCO₃, 25; KH₂PO₄, 1.18; ethylenediamine-tetra-acetic acid di-potassium salt (K₂EDTA), 0.026; and D-glucose 5.5. Third order branches of the mesenteric artery (mean internal diameter; 237±6µm, 107 vessels from 32 rats) were dissected from the mesenteric bed and cleaned of connective tissues under a light microscope. One was used immediately after dissection (day 0) whilst the others were stored separately (at 4°C, in vials containing 1ml PSS) for up to four days (day 1-day 4).

The mesenteric arterial vessel segments were mounted in a small vessel, dual chamber, myograph (chamber volume 12.5ml) for measurement of isometric tension. Following equilibration, the vessels were normalised and the viability was assessed using a standard start procedure (Chapter 2).

3.3.2. *Experimental Protocol*

Following normalisation and assessment of vessel viability the ability of the vessel to respond to contractile and dilator agonists was investigated by producing cumulative concentration response curves (CCRCs). In order to assess the effects of prolonged cold storage on vascular smooth muscle cell contractility CCRCs were obtained for the receptor-dependent vasoconstrictors noradrenaline (NA; 1×10^{-9} - 3×10^{-5} M), phenylephrine (PHE; 1×10^{-9} - 3×10^{-5} M) and endothelin-1 (ET-1; 1×10^{-11} - 3×10^{-7} M) and for the receptor-independent vasoconstrictor potassium (K^{+} ; 2.5-140mM). The use of these constrictors, which act via different mechanisms, would determine whether any alteration in contractile function following cold storage was due to altered receptor function or a general inability of the vascular smooth muscle cells to contract.

Endothelium-dependent and -independent vasodilator responses were assessed by producing CCRCs to acetylcholine (ACh; 1×10^{-9} - 3×10^{-5} M) and 3-morpholinosydnonimine (SIN-1; 1×10^{-9} - 1×10^{-4} M), respectively, in vessels submaximally precontracted with 3 μ M NA (which induced approximately 80% maximum contraction). The use of these endothelium-dependent and -independent vasodilators should determine whether prolonged cold storage altered the ability of endothelial cells to release, and/or vascular smooth muscle cells to relax in response to, endothelium-derived relaxing factors. After each CCRC the vessel was washed four times with PSS and allowed a 15 min equilibration period in PSS before exposure to the next agonist.

3.3.3. *Drugs*

All salts were obtained from BDH Laboratory Supplies (Poole, Dorset, U.K.) Acetylcholine chloride, noradrenaline bitartrate, phenylephrine hydrochloride were from Sigma (Poole, Dorset, U.K.) SIN-1 was a gift from Dr.K.Schonafinger, Cassella, Germany and endothelin-1 was obtained from Calbiochem-Novabiochem (U.K.) Ltd (Beeston, Nottingham, U.K.).

All drugs were dissolved in distilled water and stored as stock solutions at -20°C , with the exception of ET-1. Endothelin stock was dissolved in 50% ethanol. Further dilutions were made in 50% methanol/ 50% distilled water, divided into aliquots and stored as 10^{-5} M stock solutions at -20°C . On the day of use, stock solutions were thawed and subsequent dilutions were made in distilled water. These solutions were added directly to the PSS in the myograph chamber to give the final dilutions. All concentrations are expressed as final bath concentration.

3.3.4. *Statistics*

All values presented are mean \pm standard error mean (s.e.mean) for n experiments. NA, PHE, K^{+} and ET-1 -induced tension is expressed as a percentage of the maximum contractile response to that agonist. The relaxation to ACh and SIN-1 is expressed as a percentage relaxation of the initial NA-induced precontraction. Sensitivity to the agonists is expressed as the negative log of the effective concentration (M) of the drug required to produce 50% of the maximum effect (pD_2 for vasoconstrictors and $-\log\text{IC}_{50}$ for vasodilators). The sensitivity was calculated

from each concentration-response curve by fitting the Hill equation using curve fitting software (Fig.P; Biosoft, Cambridge, U.K.).

The effect of storage on maximum response and sensitivity (pD_2 , $-\log IC_{50}$) was determined by comparing responses in vessels stored for 1 to 4 days with the response produced by freshly isolated vessels using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Significance was assumed if $P < 0.05$.

3.4. Results

3.4.1. *Vasoconstrictor Responses.*

The standard start procedure demonstrated that the vessels analysed immediately following dissection were viable (i.e. produced contractions in response to a combination of NA ($10^{-5}M$) and KPSS that exceeded 13.3kPa – see Chapter 2.1.4) and that this viability was unaffected by storage over a four day period (Table 3.1.). Indeed, all the arteries used in this investigation were shown to be viable. The arteries produced strong, concentration-dependent, contractions in response to each of the vasoconstrictors, NA, PHE, KCl and ET-1. The magnitude and sensitivity of these responses were unaffected by storage (Table 3.2.).

The concentration of noradrenaline required to produce approximately 80% of the maximum contractile response in freshly isolated vessels was $3\mu M$. This concentration was used to precontract the tissues in order to assess the relaxant

Table 3.1. The effect of prolonged cold storage on the viability of rat mesenteric resistance arteries. Values are mean \pm s.e.mean, n numbers in parentheses.

VIABILITY (kPa)	
Day 0	26.33 \pm 1.78 (24)
Day 1	29.14 \pm 2.56 (22)
Day 2	27.87 \pm 2.08 (24)
Day 3	29.66 \pm 2.76 (20)
Day 4	31.42 \pm 2.55 (17)

The viability value is an indication of the effective pressure developed by the artery in response to a solution containing high concentrations of potassium (125mM) and NA (10^{-5} M), which produces a maximal contraction. It is calculated using the LaPlace relation (Effective pressure = Wall tension/ (internal circumference/ (2π))) and is used routinely to determine whether a vessel has been damaged during isolation. In the current investigation measurement of viability also provided an indication of whether prolonged storage had caused a loss of viability. Rat mesenteric arteries usually produce an effective pressure >20 kPa and are considered unviable if the effective active pressure developed in response to NA-KPSS is less than 13.3kPa (100mmHg). All the vessels mounted in the organ bath for the current study were shown to be viable.

Table 3.2. The effects of cold storage on the (a) maximum contraction (mN/mm) and (b) sensitivity (pD₂) of rat 3rd order mesenteric arteries to vasoconstrictors. Results are shown as mean \pm s.e.mean, n=10.

(a)

Maximum Contraction (mN/mm)					
	Day 0	Day 1	Day 2	Day 3	Day 4
NA	2.79 \pm 0.28	2.52 \pm 0.31	3.06 \pm 0.32	2.26 \pm 0.18	2.09 \pm 0.28
PHE	2.91 \pm 0.16	3.39 \pm 0.25	3.59 \pm 0.41	2.73 \pm 0.34	2.76 \pm 0.45
K ⁺	1.35 \pm 0.07	1.44 \pm 0.18	1.79 \pm 0.31	1.50 \pm 0.21	1.83 \pm 0.27
ET-1	2.90 \pm 0.33	3.23 \pm 0.25	2.64 \pm 0.42	2.91 \pm 0.35	2.57 \pm 0.29

(b)

Sensitivity (pD ₂)					
	Day 0	Day 1	Day 2	Day 3	Day 4
NA	5.76 \pm 0.08	5.85 \pm 0.06	5.85 \pm 0.05	5.89 \pm 0.09	5.97 \pm 0.10
PHE	5.60 \pm 0.09	5.55 \pm 0.04	5.60 \pm 0.10	5.47 \pm 0.06	5.74 \pm 0.17
K ⁺	1.58 \pm 0.04	1.64 \pm 0.01	1.62 \pm 0.04	1.61 \pm 0.04	1.54 \pm 0.04
ET-1	8.45 \pm 0.18	8.38 \pm 0.09	8.50 \pm 0.09	8.66 \pm 0.11	8.59 \pm 0.13

Comparison of stored (days 1 to 4), with freshly isolated (day 0), vessels using oneway ANOVA and Dunnett's post hoc test, demonstrated that contractile function was not significantly altered during storage.

properties of the vasodilator agents. The contraction produced by this concentration of NA was unchanged by storage for two days; an apparent reduction on days 3 and 4 (Table 3.3.) did not achieve significance.

3.4.2. *Responses to vasodilator agonists.*

Following precontraction with 3 μ M NA, ACh and SIN-1 both induced concentration-dependent relaxations which were still evident after four days of storage. Storage of the vessels for up to three days resulted in no change in the responses induced by these agonists (Table 3.4.). The magnitude and sensitivity of the relaxations to these agonists was unchanged until the fourth day of storage, when the arteries demonstrated a significant increase in the maximum response to ACh and a significant increase in sensitivity to both ACh and SIN-1 (Table 3.4.; Figures 3.1.). A trend towards increased maximum response to SIN-1 was also observed but this did not achieve significance.

3.5. Discussion

The results of this study indicate that rat mesenteric resistance arteries remain viable if stored in PSS at 4°C for up to four days. The sensitivity of the arteries to the vasodilators, ACh and SIN-1, remained stable following the first three days of cold storage and thereafter increased on day 4, whereas responses to the vasoconstrictors were unaltered.

Table 3.3. The effect of prolonged cold storage on the magnitude of noradrenaline (3 μ M)-induced contraction obtained prior to the construction of CCRC's to the vasodilators ACh and SIN-1 in rat mesenteric resistance artery.

	Contraction (mN/mm)	
	Pre-ACh	Pre-SIN-1
Day 0	1.91 \pm 0.15	2.24 \pm 0.19
Day 1	2.47 \pm 0.23	2.47 \pm 0.17
Day 2	2.07 \pm 0.20	2.31 \pm 0.16
Day 3	1.45 \pm 0.15	1.63 \pm 0.21
Day 4	1.92 \pm 0.42	1.72 \pm 0.45

Values are mean \pm s.e.mean, n=10. Comparison of stored (days 1 to 4), with freshly isolated (day 0), vessels using oneway ANOVA and Dunnett's post hoc test, demonstrated that pre-contraction was not significantly altered during storage.

Table 3.4. The effect of prolonged cold storage on (a) maximum relaxation (%) and (b) sensitivity ($-\log IC_{50}$) of rat 3rd order mesenteric arteries to vasodilators.

(a)

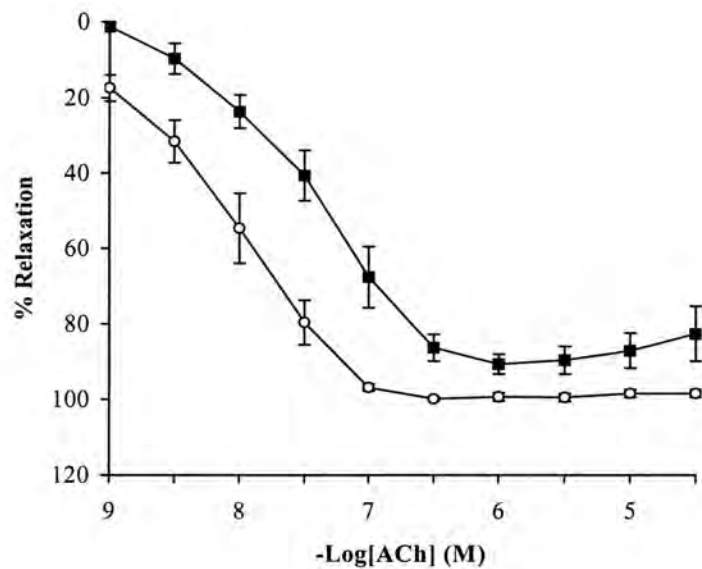
	Maximum Relaxation (%)				
	Day 0	Day 1	Day 2	Day 3	Day 4
ACh	92.65±2.84	94.28±1.96	96.06±2.79	90.97±4.82	100.36±0.36*
SIN-1	84.84±6.56	90.72±3.48	94.33±2.82	96.21±1.62	96.12±2.08

(b)

	$-\log IC_{50}$				
	Day 0	Day 1	Day 2	Day 3	Day 4
ACh	7.46±0.13	7.26±0.06	7.27±0.16	7.41±0.12	7.97±0.11*
SIN-1	4.87±0.10	5.13±0.18	4.88±0.10	5.07±0.10	5.52±0.08†

Values are mean \pm s.e.mean, n=10. * $P<0.05$ when compared with maximum response and $-\log IC_{50}$ for day 0 (ACh) and † $P<0.01$ when compared with $-\log IC_{50}$ value for day 0 (SIN-1), using one-way ANOVA followed by Dunnett's post test.

(a) Acetylcholine



(b) SIN-1

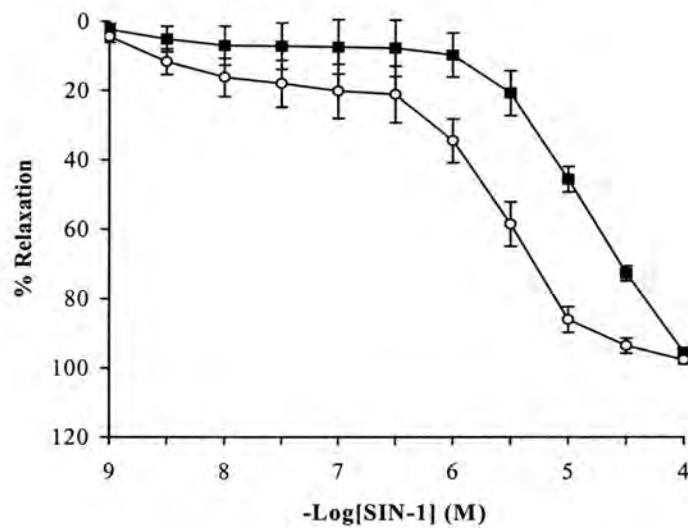


Figure 3.1. The effect of cold storage on the cumulative concentration response curves to (a) acetylcholine (ACh; 10^{-9} - 3×10^{-5} M) and (b) 3-morpholinosydnonimine (SIN-1; 10^{-9} - 10^{-4} M) in rat mesenteric resistance arteries. Each point represents mean \pm s.e. mean, $n=10$. ■ Day 0, ○ Day 4.

3.5.1. *Vasodilator Function.*

Previous investigations have shown that cells in the vascular wall undergo time-dependent alterations when stored at 4°C. The severity and speed of these changes vary between different cell types with the earliest alterations occurring in nerve fibres and endothelial cells (Kristek *et al.*, 1993). These morphological alterations are accompanied by functional abnormalities, with endothelium-dependent relaxation reduced after as little as 24h storage (Flanders *et al.*, 1996). Similarly, cryopreservation, which involves storing the tissues in DMSO at either -70 or -190°C, produces endothelial cell dysfunction (Ku *et al.* 1990). In addition to the problems this poses for pharmacological investigation, such endothelial cell dysfunction is a cause for concern for bypass grafting and organ transplantation. Consequently the preservative effects of a variety of solutions have been investigated. Whilst this has produced evidence that some preservation solutions (University of Wisconsin, St Thomas' Hospital) are better for preserving endothelial function in rabbit aorta (Eberl *et al.*, 1993) and porcine hepatic artery (Flanders *et al.*, 1996), the reasons for these differences are unclear. Indications into the protective action of these solutions are not provided by comparison of their contents. University of Wisconsin (Flanders *et al.*, 1996) solution is a calcium-free, high potassium solution which contains agents which inhibit free radical generation (adenosine, allopurinol, glutathione) and platelet aggregation (adenosine). In contrast, St. Thomas' solution is a simple salt solution containing moderately high concentrations of potassium and magnesium but no glucose (Eberl *et al.*, 1993).

In contrast to investigations using large conduit arteries, these results demonstrated that the rat mesenteric resistance artery retains endothelium-dependent vasodilator function throughout the period of preservation. This suggests that the endothelium in these vessels does not become damaged as rapidly as the endothelium of rabbit aortae. Indeed, the sensitivity to both endothelium-dependent and -independent dilators was increased significantly after storage for four days.

In rat mesenteric resistance arteries ACh causes relaxation by stimulating the release of NO (following activation of constitutive NO synthase (eNOS; Forstermann *et al.*, 1991)) and a hyperpolarizing factor (EDHF) from the endothelium (Furchgott *et al.*, 1987, Feelisch & Noack, 1987). NO elicits vasodilatation by stimulation of soluble guanylate cyclase, resulting in elevation of cyclic guanosine 3',5' monophosphate (cGMP; Waldman & Murad, 1987) which lowers intracellular Ca^{2+} concentrations in the smooth muscle cells. In some vessels NO can also cause relaxation by smooth muscle hyperpolarisation, achieved by stimulating ATP-sensitive and/ or charybdotoxin-sensitive potassium channels (Plane *et al.*, 1996; Plane *et al.*, 1998). Hence, alterations in the production, release or action of either or both of these mediators may account for the increase in sensitivity to ACh on day 4. One possible explanation for the observed increase in sensitivity to ACh is that guanylate cyclase-mediated relaxation has been upregulated in stored arteries (Moncada *et al.*, 1991).

It has been demonstrated that the basal release of NO from the endothelium *in vivo* reduces the sensitivity of the vascular wall to agonist-induced dilatation, probably at a site distal to soluble guanylate cyclase (Busse *et al.*, 1989). This basal release is

stimulated by shear stress (Nishida *et al.*, 1992) and, therefore, is unlikely to occur in the stored arteries. Consequently, it is possible that a reduced basal release of endothelium-derived NO results in an upregulation of guanylate cyclase-mediated relaxation. It should be noted, however, that endothelial cell dysfunction in stored rabbit aorta was not accompanied by an increased sensitivity to sodium nitroprusside (Torok *et al.*, 1993). Alternatively, the increased sensitivity observed to ACh could be due to an increase in permeability of the vascular smooth muscle cells. The hydrophobic property of NO is central to its physiological function, permitting it to pass freely across cell membranes by passive diffusion (Mann *et al.*, 2003). Previous investigations have demonstrated that increases in membrane permeability can occur as a consequence of destabilization of the membrane bilayers (Ehringer *et al.*, 2002) and it has been suggested that increased smooth muscle cell permeability may develop as a consequence of storage (Torok *et al.*, 1993). Such changes may allow more efficient access of NO to soluble guanylate cyclase in the vascular smooth muscle cells.

A large component of ACh-mediated relaxation in rat mesenteric resistance arteries is mediated by EDHF (Plane *et al.*, 1997) and, therefore, the increased sensitivity to ACh observed on day 4 may be due to an increased activity of EDHF. The mechanisms of such an increase can only be speculated upon since the identity of EDHF still remains elusive, although several possibilities can be considered. Some studies have indicated that, in the presence of insult (e.g. hypertension), EDHF-mediated relaxation is augmented to compensate for the reduced response to NO (Kagota *et al.*, 1999; Katusic, 2002), suggesting that NO activity may regulate EDHF

activity. In addition, previous studies have suggested that EDHF acts through gap junctions (Chaytoe *et al.*, 1998) and therefore it is feasible that changes in membrane properties may alter the function of gap junctions.

SIN-1, an endothelium-independent vasodilator, was included in the study to ensure that the vessels retained the ability to relax. At the time it was thought that SIN-1, the active metabolite of molsidomine, was an NO donor, spontaneously releasing NO by a free radical process following base-catalysed hydrolysis to produce endothelium-independent relaxation (Feelisch, 1991). However, during the conversion of SIN-1 superoxide anions are formed, in addition to NO, which react together to form peroxynitrite (OONO^- ; Singh *et al.*, 1999). Therefore, rather than being an NO donor, SIN-1 is now thought of as an OONO^- donor. The observed biological effects of OONO^- have been shown to be mediated by direct reaction with tissue thiols causing thiol-dependent relaxations of vascular smooth muscle (Wu *et al.*, 1994) and stimulation of platelets (Moro *et al.*, 1994). In addition cultured endothelial cells treated with OONO^- demonstrated a thiol-dependent stimulation of sGC, which subsequently increased cGMP production to levels comparable to cells treated with a NO donor (Mayer *et al.*, 1995). The observed increase in sensitivity to SIN-1 on day 4, therefore, may be due to upregulation of sGC and this may account for the observed increase in sensitivity to both ACh and SIN-1.

It is unclear why endothelial cell function is preserved in the rat mesenteric arteries but not in larger vessels stored at a similar temperature (Kristek *et al.*, 1993; Torok *et al.*, 1993; Flanders *et al.*, 1996). The size and origin of the vessels may be important

as endothelial cell function appears to vary according to the diameter of the vessel (Haeffliger *et al.*, 1993), and upon its anatomical origin (Vanhoutte & Miller, 1985). Alternatively, the different storage conditions may be significant as some preservative solutions appear to be more effective than others in maintaining the function of the endothelium (Eberl *et al.*, 1993; Vohra *et al.*, 1997). Further work is required to clarify the cause of these variations.

3.5.2. *Vasoconstrictor Function.*

The measurement of vessel viability is usually performed to ensure that vessel preparation does not damage the vessel wall. As an experienced myographer will rarely cause such damage, the inclusion of this measurement in the present study further demonstrated that prolonged storage did not impair viability. Indeed, the effective pressure (viability) measurements were remarkably consistent throughout the study (Table 3.1.).

It has been suggested that the changes in contractile function detected in stored vessels represent a balance between the effects of endothelial cell and smooth muscle cell dysfunction (Kristek *et al.*, 1993). Consequently, the observed increase in sensitivity of the rabbit aorta to NA after preservation for three to four days (Shibata, 1969; Carrier *et al.*, 1973; Vohra *et al.*, 1997) may be the result of early endothelial cell dysfunction, as the vascular endothelium can modulate the constrictor response to many agonists. This would be similar to the increased reactivity to NA (Dohi *et al.*, 1990) documented following removal of the endothelium in mesenteric resistance arteries from normal rats. Alternatively, Kristek *et al.*, (1993) suggested

that storage leads to altered calcium homeostasis, as a result of increased permeability and membrane depolarisation. As the time of preservation lengthens the increased response to NA is probably balanced by an attenuation resulting from gradual impairment of contractile function.

This investigation demonstrated that NA and PHE-induced contractions in the rat mesenteric artery were unaffected by storage. It is possible that the conservation of endothelium-dependent relaxation prevented the increased responsiveness to these agonists reported by other investigators (Shibata, 1969; Carrier *et al.*, 1973; Kristek *et al.*, 1993). Furthermore, the maintenance of these responses indicated that α -adrenoceptor-mediated vasoconstriction was not altered by storage.

In order to assess the responses to vasodilators it is necessary to pre-contract the vessels using a submaximal concentration in order to prevent over estimation of the sensitivity of the tissue to these agents. In the present study, the submaximal concentration of NA (3×10^{-6} M) used to pre-contract the vessels often produced a biphasic response with a slight loss of tone after the initial contraction, after which the contraction stabilised. This loss of tone appeared to be more marked on days 3 and 4, and this may account for the apparent reduction in pre-contraction to NA on these days. The lack of significance, however, may be due to the variability of this biphasic response. Therefore, this demonstrates that responses to NA are maintained throughout storage, with no loss of response to the dilators.

In contrast to NA, it has previously been reported that KCl-mediated vasoconstriction is significantly reduced following preservation for four days but this reduction could be partially attenuated if preservation was performed in a high potassium Ringer solution (Shibata, 1969). This may be due to storage-induced alterations in the smooth muscle cell polarisation state as preservation has been shown to significantly reduce intracellular potassium but increase intracellular sodium and calcium (Carrier *et al.*, 1973). Our results demonstrated that such changes were not significant in the rat mesenteric artery, as responses to K^+ were unaltered.

Contractile responses to ET-1 following prolonged storage of the vessels have not been investigated previously. However, various insults, including hypoxia, can lead to the release of this potent vasoconstrictor by endothelial cells (reviewed in Haynes & Webb, 1993), resulting in receptor down-regulation and reduced sensitivity to this agonist (Clozel *et al.*, 1993). Consequently, it is possible that the endothelial cell damage reported in larger vessels following storage (Kristek *et al.*, 1993; Torok *et al.*, 1993; Flanders *et al.*, 1996), would have caused reduced sensitivity to ET-1. The unaltered response in the rat mesenteric artery to ET-1 in the present study suggests that prolonged storage of this tissue does not lead to significant release of ET-1 from the vascular endothelium. This supports the argument that there is no significant endothelial cell damage in these vessels after storage which is consistent with the results obtained using ACh.

Finally, the maintenance of the responsiveness to the vasoconstrictors studied suggests that the contractile activity of the smooth muscle was not attenuated during preservation. This is consistent with previous investigations carried out in rabbit conduit arteries in which contractile function to exogenously applied vasoconstrictors was not reduced after preservation for up to eight days (Shibata, 1969; Kristek *et al.*, 1993). Indeed, Shibata (1969) demonstrated that contractile responses to NA in those vessels were only reduced significantly after 10-12 days preservation and detectable contractions were still present after twenty days storage in Ringer's solution.

In summary, this study has clearly demonstrated that rat mesenteric artery segments maintain stable responsiveness to vasoconstrictors and to endothelium-dependent and endothelium-independent vasodilators for up to three days when stored in a simple PSS at 4°C. The increase in sensitivity observed with both ACh (endothelium-dependent) and SIN-1 (endothelium-independent) on day four suggests an increase in smooth muscle sensitivity to vasodilators after this storage period. Preservation of the contractile responses implies that receptor function and smooth muscle cell polarisation state are not adversely affected by preservation. Consequently, rat resistance artery segments can be stored for subsequent investigation and do not develop the significant endothelial cell damage previously observed in larger arteries, thereby allowing more efficient use of animal tissues in the future.

CHAPTER 4

THE EFFECT OF ELEVATED GLUCOSE AND INSULIN ON FUNCTION OF RAT MESENTERIC RESISTANCE ARTERY

4.1. Introduction

Diabetic microangiopathy and macroangiopathy are the principal causes of morbidity and mortality in patients with diabetes mellitus (Head & Fuller, 1990) but the mechanisms underlying abnormal microvascular function remain controversial. Patients with strict glycaemic control have a lower incidence of vascular complications (Diabetes Control and Complications Trial Group, 1993), implicating hyperglycaemia as an independent risk factor for the development of cardiovascular disease. The mechanism by which elevated glucose contributes to the pathogenesis of vascular dysfunction is not completely understood but there is much evidence to suggest that the action of both glucose and insulin can directly alter the function of cells in the vascular wall.

The majority of studies of vascular function in diabetic animal models have reported impaired endothelium-dependent relaxation in both resistance (Taylor *et al.*, 1992) and conduit (Durante *et al.*, 1988; Abiru *et al.*, 1990; Cameron & Cotter, 1992) arteries. This may be the result of hyperglycaemia as several groups have reported attenuated endothelium-dependent relaxation in arteries from non-diabetic animals following exposure to elevated concentrations of glucose (Teshfamarham *et al.*, 1990; Taylor & Poston, 1994). Furthermore, endothelial dysfunction does not develop in arteries from rats with streptozotocin (STZ)-induced diabetes if they are treated with insulin to control hyperglycaemia (Taylor *et al.*, 1994). It is also possible, however, that direct effects of insulin on the arterial wall affect vasodilator function as insulin causes an endothelium-dependent relaxation in isolated arteries (Han *et al.*, 1995)

and stimulates enhanced nitric oxide release by vascular endothelial cells (Walker *et al.*, 1997).

Contractile function is also altered in patients and animals with diabetes. The nature of the alteration remains controversial, however, as responses to noradrenaline (NA) have variously been reported to be reduced (Cameron & Cotter, 1992), increased (Abebe *et al.*, 1990; Harris & MacLeod, 1988), or unaltered (Fulton *et al.*, 1991) in isolated arteries. Recently, interest has been increasing in the role of the endothelium-derived vasoconstrictor endothelin-1 (ET-1) in the vascular complications of diabetes (reviewed Hopfner & Gopalakrishnan, 1999). Reports of elevated plasma (Takahashi *et al.*, 1990) and tissue (Properzi *et al.*, 1995) concentrations of ET-1 have been followed by demonstrations of impaired ET-1-mediated constriction in both the forearm of patients with type 2 diabetes (Nugent *et al.*, 1996) and in isolated aortic rings from rats with STZ-induced diabetes (Fulton *et al.*, 1991; Hodgson & King, 1992). It remains unclear, however, whether exposure to high concentrations of glucose or insulin contributes to the impaired contractile response to ET-1.

4.2. Aim

The aim of this study was to determine the effects of acute elevations of glucose or insulin *in vitro* on resistance artery function.

4.3. Methods

4.3.1. *Measurement of vascular reactivity*

Male Cob-Wistar rats (150-250g) were killed by stunning followed by cervical dislocation and the mesentery was removed and placed in PSS. Third order mesenteric arteries (mean internal diameter $218 \pm 6 \mu\text{m}$, $n=56$) were dissected free from connective tissue and mounted as ring preparations on two $40 \mu\text{m}$ intraluminal wires in a small vessel myograph for measurement of isometric tension. Arteries were bathed in PSS, maintained at 37°C and perfused with 95% O_2 ; 5% CO_2 . Following normalisation, vessel viability was assessed using a standard start procedure and the functional integrity of the endothelium was confirmed by adding acetylcholine (ACh; $10 \mu\text{M}$) to vessels submaximally contracted with $3 \mu\text{M}$ NA (see Chapter 2 for details).

4.3.2. *Experimental Protocol*

Two mesenteric arteries were isolated from each rat and exposed to one of the following protocols:-

- (i) Incubation in 5.5mM glucose or 20mM glucose for 1 hour.
- (ii) Incubation in 5.5mM glucose or 14.5mM mannitol and 5.5mM glucose for 1 hour.
- (iii) Incubation in 15mM glucose or 44mM glucose for 1 hour.
- (iv) Incubation in 20mM glucose or 14.5mM mannitol and 5.5mM glucose for 4 hours.

The incubation medium was changed every 30min and, before continuation of the protocol, the arteries were contracted for 3min with NA-K to confirm viability.

Cumulative concentration-response curves (CCRCs) were then obtained for the receptor-dependent agonists NA (1×10^{-9} - 3×10^{-5} M), and ET-1 (1×10^{-11} - 3×10^{-7} M) and for the receptor-independent vasoconstrictor potassium (K^+ ; 2.5-140mM). Endothelium-dependent and -independent vasodilatation was assessed using the vasodilators ACh (1×10^{-9} - 3×10^{-5} M), the Ca^{2+} ionophore A23187 (1×10^{-9} - 1×10^{-5} M) and 3-morpholinysydnonimine (SIN-1; 1×10^{-9} - 1×10^{-4} M), respectively. Vasodilator responses were obtained in arteries following submaximal contraction with NA (3μ M). The glucose/mannitol remained in the bath for the duration of both the contraction and relaxation phases. After each CCRC, the contents of the bath were replaced four times with fresh PSS, containing the appropriate concentrations of glucose/mannitol, and a 20min washout period was allowed between each CCRC.

In order to investigate the role of the endothelium, some experiments were performed in arteries denuded of endothelium. The endothelium was removed by rubbing the lumen with a human hair (see Chapter 2 for details), and complete denudation was confirmed by the lack of relaxation in response to ACh (10μ M) in vessels submaximally precontracted with NA (3μ M).

The influence of insulin on vascular function was assessed separately. Two vessels were taken from each rat and incubated for 1 hour in either normal PSS (5.5mM glucose) or in PSS containing insulin (1000mU/l). These vessels were then exposed to the protocol above. The insulin remained in the organ bath for the duration of the experiment.

4.3.3. *Drugs*

Reagents for the preparation of PSS were obtained from BDH Laboratory Supplies, Poole, Dorset, UK. Acetylcholine chloride, A23187 free acid and noradrenaline bitartrate were purchased from Sigma, Poole, Dorset, UK. SIN-1 and ET-1 were obtained from Alexis Corporation (UK) Ltd, Bingham, Nottingham. Insulin (human Velosulin 100U/ ml) was obtained from NovoNordisk, Crawley, West Sussex, U.K.

All drugs were dissolved in distilled water with the exceptions of A23187 and ET-1. A23187 was dissolved in 100% ethanol and further dilutions were made in 10% ethanol, divided into aliquots and stored as 10^{-4} M stock solution at -20°C . ET-1 was dissolved in 50% methanol and stored as 10^{-5} M stock solution at -20°C . In both cases final bath concentrations of ethanol were less than 1%. Stock solutions were thawed as required, subsequent dilutions were made in distilled water and any residual solution was discarded at the end of the experiment. All concentrations are expressed as the final molar concentration in the organ bath.

4.3.4. *Statistics*

All values presented are mean \pm standard error mean (s.e.mean) for n experiments. NA, K^{+} and ET-1-induced contractions are expressed as a percentage of the maximum contractile response to that agonist. The relaxation to ACh, A23187 and SIN-1 is expressed as a percentage relaxation of the initial NA-induced precontraction.

Sensitivity to the agonists is expressed as the negative log of the effective concentration (M) of the drug required to produce 50% of the maximum effect (pD_2 for vasoconstrictors and $-\log IC_{50}$ for vasodilators). The sensitivity was calculated from each concentration-response curve by fitting the data to the Hill equation using curve fitting software (Fig.P, Biosoft, Cambridge, UK). The effect of increased concentrations of glucose or mannitol on vascular function was assessed by comparison with normal glucose (5.5mM) controls using one-way ANOVA followed by Dunnett's post-hoc test or Kruskal-Wallis test followed by Dunn's multiple comparison test, as appropriate. The influence of 4 hour exposure to 20mM glucose was assessed by comparison with concomitant controls exposed to 14.5mM mannitol and 5.5mM glucose using Student's unpaired t-test. The influence of insulin was determined by comparison with concomitant controls using Student's unpaired t-test. In all tests, significance was assumed if $P < 0.05$.

4.4. Results

4.4.1. *Effect of elevated concentrations of glucose or mannitol*

There was no significant change in the contractile responses of vessels following 1 hour incubation in an elevated concentration of mannitol compared to 5.5mM glucose (Table 4.1a).

Incubation in 15 or 44mM glucose for 1 hour resulted in reduced sensitivity to ET-1, causing a shift to the right of the ET-1 concentration-response curve (Figure 4.1a.). This decrease in responsiveness was not evident in intact (Table 4.1) or denuded

Table 4.1. Maximum contraction (Emax., mN/mm) and sensitivity (pD₂) to vasoconstrictor agonists in rat mesenteric resistance arteries following (a) 1 hr or (b) 4 hr incubation in normal PSS or PSS containing elevated concentrations of glucose or mannitol.

(a) 1 hr

Incubation Media	NA		KCl		ET-1	
	Emax	pD ₂	Emax	pD ₂	Emax	pD ₂
5.5mM glucose (11-13)	2.57±0.28	6.06±0.12	1.57±0.24	1.58±0.02	2.63±0.31	8.66±0.08
15mM glucose (10)	2.64±0.29	5.68±0.08	2.18±0.31	1.65±0.03	2.41±0.26	8.02±0.12**
20mM glucose (9-10)	2.52±0.35	5.80±0.18	1.96±0.30	1.64±0.04	2.67±0.43	8.31±0.14
44mM glucose (10)	2.10±0.28	5.67±0.09*	1.53±0.31	1.54±0.05	1.96±0.31	8.11±0.10**
20mM mannitol (6-9)	3.03±0.29	6.10±0.08	2.27±0.28	1.57±0.06	2.55±0.45	8.78±0.15

(b) 4 hr

Incubation Media	NA		KCl		ET-1	
	Emax	pD ₂	Emax	pD ₂	Emax	pD ₂
20mM glucose (5)	2.83±0.53	5.92±0.17	2.27±0.50	1.66±0.05	2.77±0.37	8.73±0.27
20mM mannitol (4-6)	2.77±0.50	5.98±0.06	2.60±0.64	1.59±0.01	1.97±0.41	8.32±0.34

Values are expressed as mean ± s.e.mean (n values shown in parentheses). **P*<0.05, ***P*<0.01 when compared with 5.5mM glucose using one-way ANOVA followed by Dunnett's post-hoc test. NA, noradrenaline; ET-1, endothelin-1.

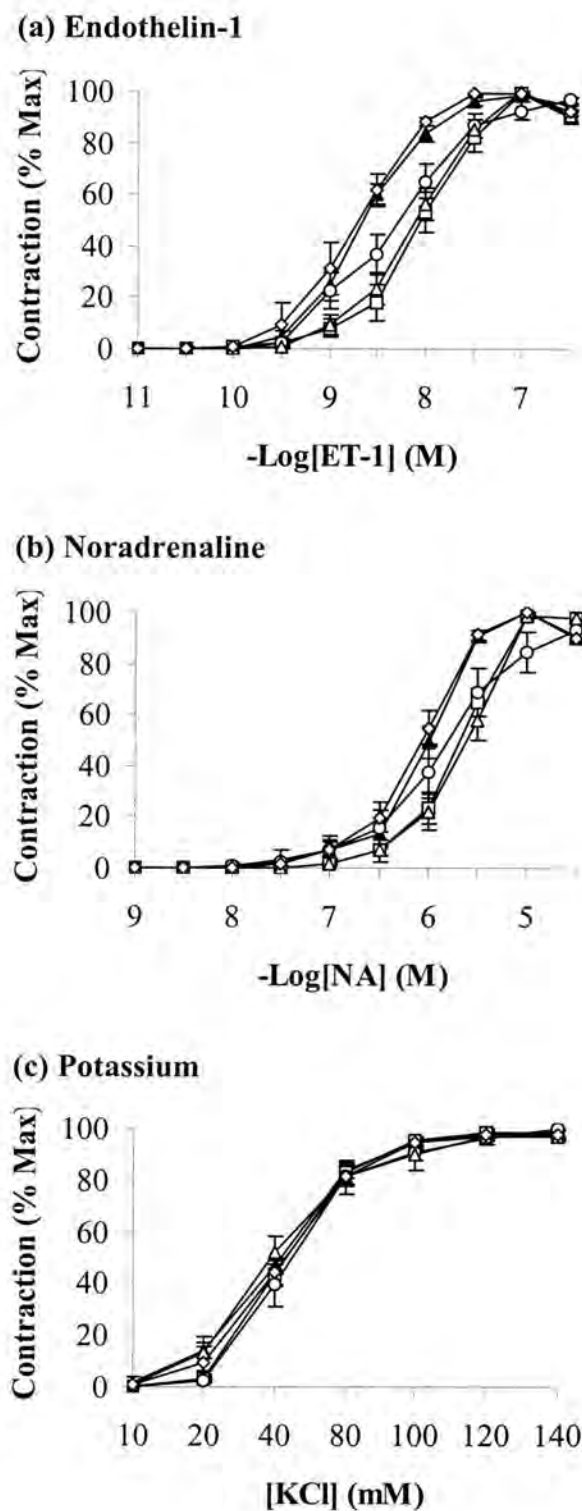


Figure 4.1. Concentration-response curves to (a) endothelin-1, (b) noradrenaline and (c) potassium in rat mesenteric resistance arteries following 1hr incubation in normal PSS (5.5mM (\blacktriangle), $n=11-13$) or PSS containing elevated concentrations of glucose (15mM (\square), $n=10$; 20mM (\circ), $n=9-10$; 44mM (\triangle), $n=10$) or mannitol (20mM (\diamond), $n=6-9$). Each point represents mean \pm s.e.mean.

(pD₂ 8.66±0.08 (n=11) vs 8.86±0.09 (n=8), *P*=0.12; Emax 2.63±0.31 (n=11) vs 2.98±0.30 (n=8), *P*=0.45) arteries exposed to 20mM glucose.

The sensitivity of the arteries to NA was significantly reduced following 1hr incubation in 44mM glucose. Despite an apparent rightward shift in the concentration response curve to NA in the presence of 15 or 20mM glucose, the alteration in sensitivity did not achieve significance (Figure 4.1b.).

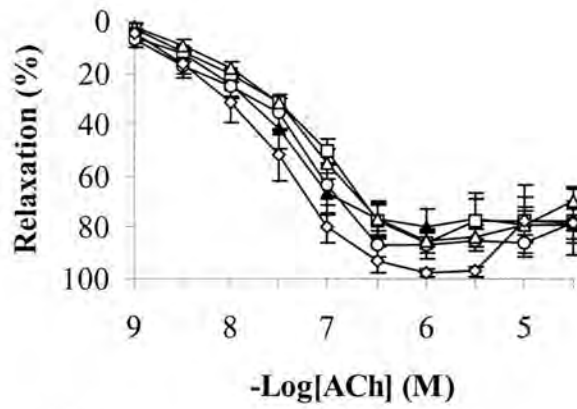
Sensitivity to K⁺ was unaffected following exposure to raised glucose concentrations (Figure 4.1c.). The magnitude of the responses to all constrictor agonists were not altered following 1 hour incubation in elevated concentrations of glucose compared with controls (Table 4.1a.).

All the arteries studied relaxed in a concentration-dependent manner in response to each of the vasodilators: ACh, A23187 and SIN-1 (Figure 4.2.). The magnitude and sensitivity of these responses were not altered following exposure to elevated concentrations of glucose compared with controls (Table 4.2.).

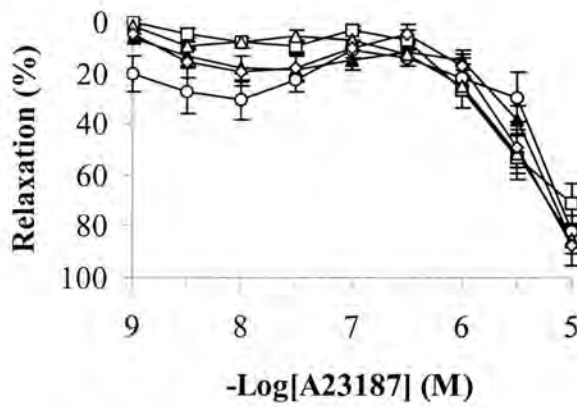
4.4.2. *Effect of Incubation Time*

There was no significant difference in either the magnitude or sensitivity of contractile or relaxant responses of vessels following 4 hours incubation in 20mM glucose compared with 14.5mM mannitol and 5.5mM glucose (Table 4.1b and Table 4.2b).

(a) Acetylcholine



(b) A23187



(c) SIN-1

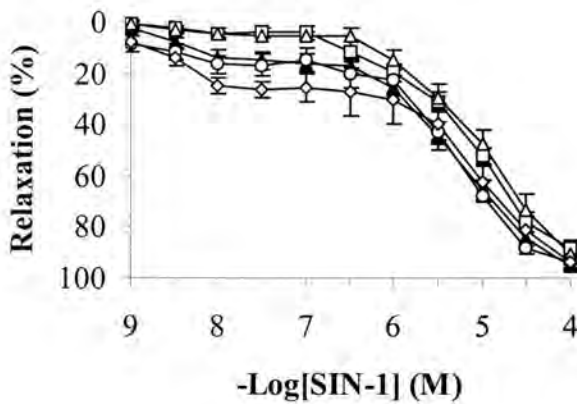


Figure 4.2. Concentration-response curves to (a) acetylcholine, (b) A23187 and (c) 3'-morpholinysydnonimine (SIN-1) in rat mesenteric resistance arteries following 1hr incubation in normal PSS (5.5mM (\blacktriangle), $n=11-13$) or PSS containing elevated concentrations of glucose (15mM (\square), $n=10$; 20mM (\circ), $n=6-10$; 44mM (\triangle), $n=8-10$) or mannitol (20mM (\diamond), $n=5-9$). Each point represents mean \pm s.e.mean and is expressed as % relaxation of $3\mu\text{M}$ NA-induced tone.

Table 4.2. Maximum relaxation (Emax, % reversal of 3 μ M NA-induced precontraction) and sensitivity (-logIC₅₀) to vasodilator agonists in rat mesenteric resistance arteries following (a) 1hr or (b) 4hr incubation in normal PSS or PSS containing elevated concentrations of glucose or mannitol.

(a) 1 hr

Incubation Media	ACh		A23187		SIN-1	
	Emax	-logIC ₅₀	Emax	-logIC ₅₀	Emax	-logIC ₅₀
5.5mM glucose (11-13)	88.61 \pm 3.66	7.02 \pm 0.24	85.62 \pm 2.24	5.41 \pm 0.05	95.62 \pm 1.22	5.28 \pm 0.12
15mM glucose (10)	88.41 \pm 5.64	7.07 \pm 0.05	70.88 \pm 7.50	5.66 \pm 0.15	88.19 \pm 3.27	5.08 \pm 0.06
20mM glucose (6-10)	93.38 \pm 2.99	7.07 \pm 0.18	81.90 \pm 6.25	5.29 \pm 0.12	93.90 \pm 1.89	5.24 \pm 0.08
44mM glucose (8-10)	91.32 \pm 2.27	6.94 \pm 0.20	80.14 \pm 4.66	5.74 \pm 0.15	90.36 \pm 3.39	4.95 \pm 0.14
20mM mannitol (5-9)	98.59 \pm 0.96	7.56 \pm 0.17	87.57 \pm 7.45	5.38 \pm 0.10	93.96 \pm 3.36	5.32 \pm 0.44

(b) 4 hr

Incubation Media	ACh		A23187		SIN-1	
	Emax	-logIC ₅₀	Emax	-logIC ₅₀	Emax	-logIC ₅₀
20mM glucose (4)	83.11 \pm 10.1	7.49 \pm 0.30	76.26 \pm 5.64	5.33 \pm 0.06	89.20 \pm 3.73	5.00 \pm 0.15
20mM mannitol (4-6)	98.47 \pm 3.61	7.48 \pm 0.09	87.12 \pm 4.43	5.49 \pm 0.19	96.36 \pm 1.94	5.48 \pm 0.12

Values are expressed as mean \pm s.e.mean (n). ACh, acetylcholine; SIN-1, 3'-morpholiniosydnonimine. NS difference between Emax or -logIC₅₀ obtained in 5.5mM glucose compared with elevated glucose (15, 20, 44mM) or mannitol (20mM).

4.4.3. *Effect of Elevated Concentrations of Insulin*

Exposure to an elevated concentration of insulin for 1hr did not alter either the size or sensitivity of vasoconstrictor responses to ET-1 NA or K⁺ (Figure 4.3. and Table 4.3.).

Likewise, the magnitude and sensitivity of vasodilator responses to ACh, A23187 and SIN-1 were not altered following exposure to elevated concentrations of insulin compared with controls (Figure 4.4 and Table 4.4).

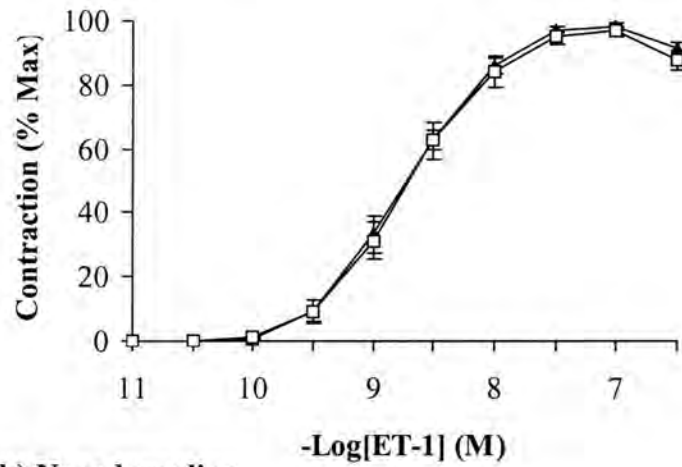
4.5. Discussion

This study assessed the time- and concentration-dependent effects of exposure to elevated concentrations of glucose or insulin on vascular contraction and relaxation. Exposure to elevated concentrations of glucose or insulin had no effect on endothelium-dependent or -independent dilatation. However, 1 hour exposure to 15 and 44mM glucose altered the sensitivity to ET-1 and NA-mediated contraction. Increasing the duration of exposure to 4 hours incubation with 20mM glucose had no further effect on contractile function. The contractile responses were unaltered following incubation with insulin.

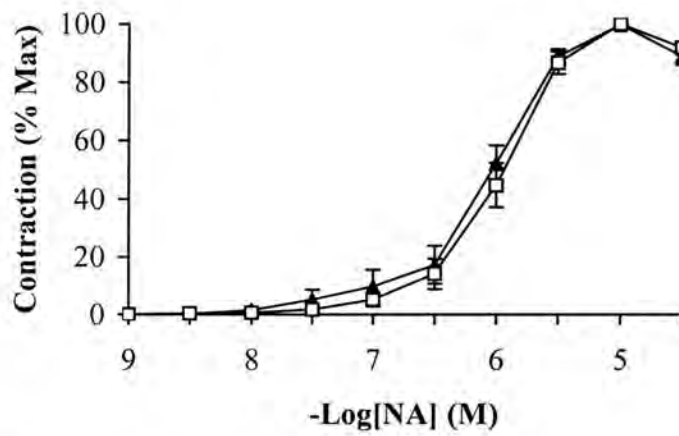
4.5.1. Effect of elevated glucose concentrations.

This study demonstrated that incubation of rat mesenteric resistance arteries in 15 or 44mM glucose for 1hr reduced their sensitivity to the vasoconstrictor peptide ET-1. This is not due to a hyperosmotic effect as an identical concentration of mannitol had

(a) Endothelin-1



(b) Noradrenaline



(c) Potassium

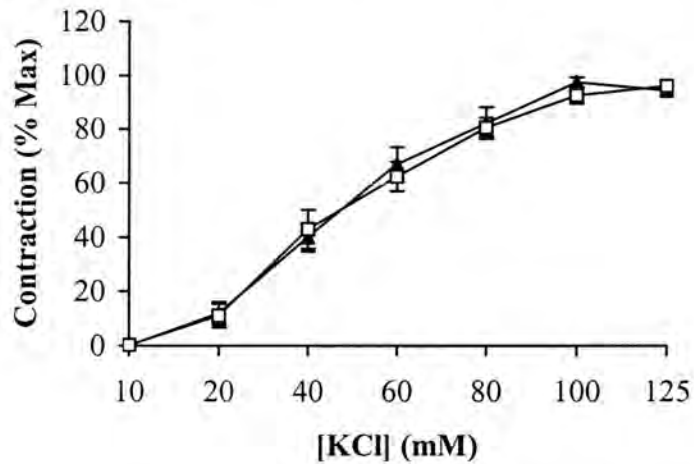


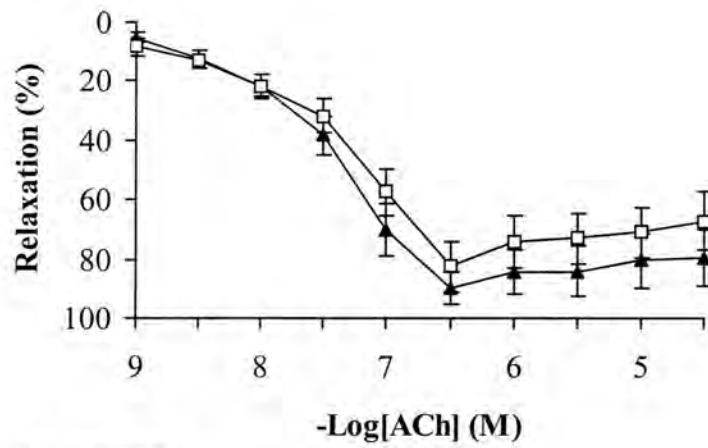
Figure 4.3. Concentration-response curves to the vasoconstrictors (a) endothelin-1, (b) noradrenaline and (c) K^+ in rat mesenteric resistance arteries following 1hr incubation in normal PSS (▲, n=10-12) or PSS containing an elevated concentration (1000mU/l) of insulin (□, n=10-12) Each point represents mean \pm s.e.mean.

Table 4.3. Maximum contraction (Emax., mN/mm) and sensitivity (pD₂) to vasoconstrictor agonists in rat mesenteric resistance arteries following incubation in normal PSS or PSS containing an elevated concentration of insulin (1000mU/l).

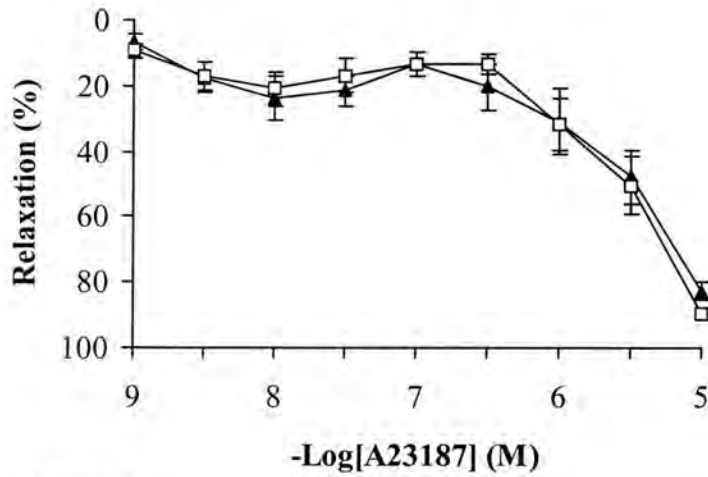
	NA		K ⁺		ET-1	
	Emax.	pD ₂	Emax.	pD ₂	Emax.	pD ₂
PSS (10-12)	2.42±0.25	6.10±0.13	1.84±0.21	1.67±0.05	2.21±0.28	8.80±0.09
Insulin (10-12)	2.93±0.20	5.97±0.09	1.88±0.19	1.67±0.05	2.82±0.26	8.68±0.10

Values are expressed as mean ± s.e.mean (n). NA, noradrenaline; ET-1, endothelin-1.

(a) Acetylcholine



(b) A23187



(c) SIN-1

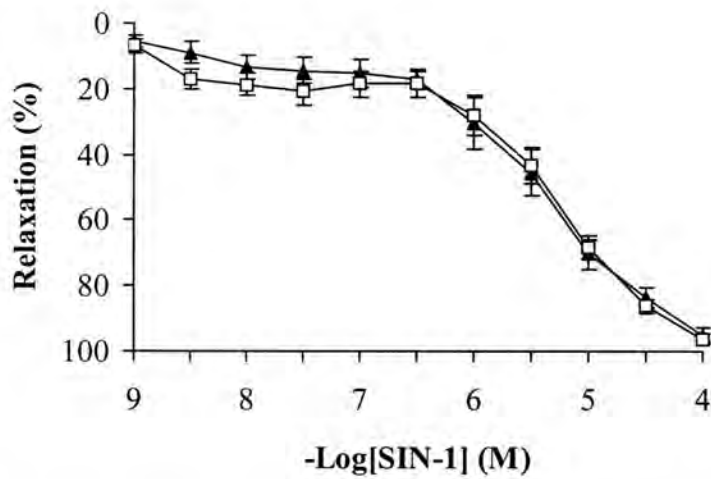


Figure 4.4. Concentration-response curves to the vasodilators (a) acetylcholine, (b) A23187 and (c) 3'-morpholinysydnonimine (SIN-1) in rat mesenteric resistance arteries following 1hr incubation in normal PSS (\blacktriangle , $n=10-12$) or PSS containing 1000mU/l of insulin (\square , $n=10-12$) Each point represents mean \pm s.e.mean.

Table 4.4. Maximum relaxation (Emax., % reversal of 3μM NA-induced precontraction) and sensitivity (-logIC₅₀) to vasodilator agonists in rat mesenteric resistance arteries following 1hr incubation in normal PSS or PSS containing an elevated concentration of insulin (1000mU/l).

	ACh		A23187		SIN-1	
	Emax.	-logIC ₅₀	Emax.	-logIC ₅₀	Emax.	-logIC ₅₀
Normal (10-12)	96.43±2.73	7.23±0.13	83.36±2.74	5.66±0.17	94.95±2.03	5.37±0.17
Insulin (10-12)	90.86±2.90	7.11±0.16	89.63±1.88	5.55±0.11	96.12±1.32	5.30±0.10

Values are expressed as mean ± s.e.mean (n). ACh, acetylcholine; SIN-1, 3'-morpholinisydnonimine. NS difference between the Emax or -logIC₅₀ obtained in normal medium or that containing insulin for any agonist.

no effect. A non-specific impairment of vascular smooth muscle cell contractility is also unlikely since responses to K^+ (direct depolarising agent) were unaltered.

The impaired sensitivity to ET-1 following incubation in 15 or 44mM glucose is consistent with reports of attenuated ET-1-mediated contraction in the forearm of patients with Type 2 diabetes (Nugent *et al.*, 1996) and in aortic rings isolated from rats with STZ-induced diabetes (Fulton *et al.*, 1991; Hodgson & King, 1992). It is not clear why 20mM glucose had no effect. High glucose concentrations can trigger the release of ET-1 by endothelial cells (Yamauchi *et al.*, 1990), which may account for the raised plasma concentrations of this peptide in diabetic patients (Takahashi *et al.*, 1990) and for its increased release from mesenteric arteries of diabetic rats (Takeda *et al.*, 1991). Although increased glucose concentrations could impair contractile responses to ET-1 by increasing production of endothelium-derived vasodilators such as nitric oxide (NO) and prostacyclin (PGI_2 ; Wanner *et al.*, 1989; DeNucci *et al.*, 1988), this hypothesis is not supported by investigations using aortae from diabetic rats (Hodgson & King, 1992) and retinal pericytes cultured in the presence of elevated glucose (Chakravarthy *et al.*, 1994), which demonstrated that the impaired response to ET-1 was endothelium-independent. In the present study, however, an impaired contractile response to ET-1 was not observed in endothelium-denuded vessels. However, as investigations with denuded vessels were only performed in 20mM glucose the influence of the endothelium on the reduced response to ET-1 in the presence of 15 or 44mM glucose is not clear.

Alternatively, the impaired response to ET-1 could result from ET_A receptor down-regulation (Awazu *et al.*, 1991; Miasiro & Paiva, 1990), due to raised concentrations

of ET-1 (Clozel *et al.*, 1993), a mechanism which has been implicated in the reduced response to ET-1 in aortae from STZ-induced diabetic rats (Hodgson & King, 1992). However, the ability of glucose to stimulate ET-1 production from endothelial cells *in vitro* is only evident after 3hrs (Yamauchi *et al.*, 1990) and therefore the time course used currently (1hr) may be insufficient to induce down-regulation of ET-1 receptors.

Vasodilatation in response to ACh, which induces relaxation by a receptor-dependent release of NO and endothelium-derived relaxing factor (EDHF) from the endothelial cells, has been reported to be impaired both in patients (McNally *et al.*, 1994) and in animals with diabetes (Taylor *et al.*, 1992; Durante *et al.*, 1988; Abiru *et al.*, 1990; Cameron & Cotter, 1992). Exposing arteries from non-diabetic animals to raised glucose concentrations *in vitro* (Tesfamariam *et al.*, 1990; Taylor & Poston, 1994) can induce a similar attenuation. The glucose-mediated alteration appears to be the result of alterations to the muscarinic receptor as responses to a receptor-independent, endothelium-dependent vasodilator (the calcium ionophore, A23187) and to a NO donor (sodium nitroprusside) were unaffected (Tesfamariam *et al.*, 1990). In the present investigation, however, ACh-induced relaxation, as well as responses to A23187 and SIN-1, was unaffected by incubation in the presence of high glucose concentrations.

4.5.2. Effect of Incubation Time

The results of this study demonstrate that increasing glucose exposure time from 1 hour to 4 hours had no effect on either the contractile or relaxant responses. It is

possible, however, that this is a type 2 error as the groups used for this comparison were small. The effect of time was not pursued in these experiments as the decision had been taken to use a 1 hour incubation with the human vessels. This contrasts with those reported previously (Tesfamariam *et al.*, 1990) which have demonstrated that glucose-mediated attenuation of endothelium-dependent relaxation is a time-dependent phenomenon; the abnormality produced in isolated rabbit aortae is less pronounced after a 2-3 hour incubation than after a 6 hour exposure to elevated glucose (Tesfamariam *et al.*, 1990). Therefore, the maintained endothelial cell function in our investigation may be due to the shorter (1 and 4 hr) incubation periods used. This conclusion is supported by the studies of Heygate *et al* (1993) who demonstrated that endothelium-dependent vasodilatation of rat mesenteric resistance arteries was only reduced after 6hrs incubation in 40mM glucose.

4.5.3. Effect of elevated concentrations of insulin

In contrast to the effect of elevated glucose, incubation of rat mesenteric resistance arteries in PSS containing an elevated concentration of insulin (1000mU/l) had no effect on the contractions induced by ET-1, NA or K⁺. This was unexpected as insulin has direct vasoactive properties and, in previous studies, caused vasodilatation in the human forearm *in vivo* (Anderson *et al.*, 1991; Creager *et al.*, 1985; Sakai *et al.*, 1993) and inhibited vasoconstriction by a number of agonists in isolated arteries *in vitro* (Alexander & Oake, 1977; Wu *et al.*, 1994; Yagi *et al.*, 1988; McNally *et al.*, 1995). Previous investigations with isolated rat mesenteric arteries have reported attenuation of NA-induced vasoconstriction in the presence of insulin (Walker *et al.*, 1997; Wambach & Liu, 1991), although one of these studies

only observed a significant effect at insulin concentrations greater than 100U/l (Wambach & Liu, 1991). The use of different incubation periods is unlikely to account for the different results obtained in the current investigation since the action of insulin, whilst known to be concentration-dependent, is not time-dependent (McNally *et al.*, 1995).

Exposure to high concentrations of insulin were expected to alter ET-1-mediated contraction as insulin can augment both acute and chronic ET-1 secretion (Hu *et al.*, 1993; Hattori *et al.*, 1991; Oliver *et al.*, 1991) and stimulate the production of ET-1 (Hu *et al.*, 1993; Oliver *et al.*, 1991) *in vitro*. However, the fact that insulin-mediated release of ET-1 from cultured endothelial cells only occurs at supraphysiological concentrations (25,000mU/l) may explain the absence of a significant effect in the current investigation which used a significantly lower concentration (1000mU/l) of insulin.

The unaltered responses to endothelium-dependent vasodilators following incubation with insulin is consistent with previous investigations using isolated resistance arteries incubated *in vitro* in a similar fashion (Walker *et al.*, 1997; McNally *et al.*, 1995). As insulin treatment *in vivo* prevents the development of a defect in endothelium-dependent relaxation in diabetic rats (Taylor *et al.*, 1994), these results suggest that insulin may act by reducing the circulating glucose concentrations rather than by directly altering the cells of the arterial wall. This appears to be confirmed by the recent demonstration that endothelium-dependent relaxation in aortic rings from

STZ-induced diabetic rats is improved by insulin when administered *in vivo* but not when administered *in vitro* (Pieper, 1997).

In summary, these results demonstrate that acute elevation of glucose to a pathophysiologically relevant concentration (15mM), but not insulin, reduces the vascular sensitivity to ET-1 in the rat mesenteric resistance artery *in vitro*. This suggests that elevated glucose concentrations may be a primary factor for the impaired contractile response to ET-1 in diabetic patients and this may contribute to the development of the vascular complications of diabetes. The mechanisms underlying the impaired response to ET-1 are unclear and future studies using endothelin receptor antagonists and NO synthase inhibitors may be beneficial in determining the relative contributions of receptor down-regulation and increased vasodilator production by the vascular wall.

CHAPTER 5

FUNCTIONAL AND MORPHOLOGICAL

CHARACTERISTICS OF VESSELS

ISOLATED FROM HUMAN SUBCUTANEOUS

FAT BIOPSIES.

5.1. Introduction

Isolated vessel techniques are used extensively to investigate mechanisms associated with the development of a variety of different disease processes. The majority of studies use vessels isolated from experimental animals but the results from such studies are sometimes difficult to extrapolate to humans due to the heterogeneity of conditions used: for example, species differences, disease duration, variations between different vessel types or the method of study. In order to overcome these problems a technique (small vessel myography) was developed to obtain small arteries from subcutaneous fat for investigation of vascular function (Aalkjaer *et al.*, 1986). This technique enables the intrinsic properties of isolated human resistance arteries, which contribute significantly to the maintenance of blood pressure, to be studied from patients and matched control subjects.

The vascular endothelium can modulate vascular tone by releasing various factors, including nitric oxide (NO), prostacyclin (PGI₂) and endothelium-derived hyperpolarising factor (EDHF) (Vanhoutte, 1989). It is recognised that functional responses differ between arteries from a variety of anatomical locations, as a result of heterogeneity in receptor populations and endothelium-derived relaxing factors (Vanhoutte & Miller, 1985). Receptor populations may vary in proximal and distal sections of the same artery (Ohkubo & Chiba, 1988) and there is preliminary evidence to suggest that acetylcholine (ACh)-induced endothelium-dependent relaxation may differ in small and large resistance arteries taken from human subcutaneous fat (Angus *et al.*, 1992).

The relative contributions of NO, PGI₂ and EDHF to endothelium-dependent relaxation also appear to depend both upon the origin and the size of a particular vessel (Shimokawa *et al.*, 1996; Urakami-Harasawa *et al.*, 1997). In a variety of arteries, particularly those with a small diameter, a component of the endothelium-dependent relaxation is insensitive to NO synthase and cyclooxygenase inhibition. This NO- and prostanoid-independent relaxation is accompanied by an endothelium-dependent hyperpolarisation of the vascular smooth muscle cell membrane potential suggesting the existence of an EDHF (Cohen & Vanhoutte, 1995), the identity of which remains elusive. The contribution of EDHF has been investigated by increasing extracellular potassium concentrations or by using a variety of potassium channel antagonists. EDHF-mediated relaxation is attenuated by either apamin (a specific inhibitor of small conductance (SK_{Ca}) calcium-activated potassium channels) alone or in combination with charybdotoxin (ChTx; a non-specific inhibitor of calcium-activated potassium channels) (Murphy & Brayden, 1995; Corriu *et al.*, 1996; Zygmunt & Högestätt, 1996).

To date, however, there have been relatively few investigations of heterogeneity of vessel type and endothelium-dependent relaxation in human arteries. Likewise, few studies have investigated the contributions of PGI₂, NO and EDHF(s) to endothelium-dependent relaxation in human resistance arteries (Deng *et al.*, 1995; Hillier *et al.*, 1998; Ohlmann *et al.*, 1997; Petersson *et al.*, 1995; Urakami-Harasawa *et al.*, 1997; Wallerstedt & Bodelsson, 1997). These studies have suggested that all three factors may contribute to relaxation of human resistance arteries but comparisons are difficult as they have used arteries from a variety of anatomical locations (omental, gluteal, pial, gastroepiploic). In addition, experimental protocols have varied with the choice of

different vasodilators (ACh, bradykinin (BK), A23187, substance P) and the use of several different vasoconstrictors (noradrenaline (NA), prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$), U46619) to pre-contract the vessels.

5.2. Aims

Therefore, the aims of this study were (1) to determine criteria for identifying differences between vessels isolated from biopsies of subcutaneous fat resistance using gross morphological, histological and functional procedures and (2) to identify the mediators of ACh-induced endothelium-dependent relaxation of NA-contracted human subcutaneous resistance arteries. NA was chosen as the vasoconstrictor since it is the best characterised physiological agent both *in vitro* and *in vivo*.

5.3. Methods

5.3.1. Subjects

Gluteal biopsies of skin and subcutaneous fat (approximately 2cm x 1cm x 1cm) were obtained under local anaesthesia (2% lignocaine hydrochloride; Astra, Hertfordshire, U.K.) from 10 normotensive, non-smoking, male volunteers (age 56±3 yrs) and immediately placed in physiological salt solution (PSS). All subjects gave written informed consent and the protocol was approved by the Lothian Research Ethics Committee. Vessels were dissected from these biopsies using light microscopy and were identified as either small or large resistance arteries or veins by differences in their physical characteristics. The wall of the vein is very thin relative to the diameter of the vessel and, when dissected from the biopsy, the vein collapses in on itself. In contrast, the thickness of the artery wall is similar to the diameter of the vessel and the artery

maintains its shape when dissected free from surrounding connective tissue. The third type of vessel, appears larger than resistance-sized arteries, and combines physical characteristics of arteries and veins. From the 10 biopsies taken, 2 contained all three types of vessels. Resistance and large arteries were isolated from 5 of the biopsies, whilst the remaining 3 biopsies contained only veins.

5.3.2. *Morphological Investigations*

Human subcutaneous vessels were dissected free of fat and fixed in 10% neutral buffered formalin for 24hrs at room temperature. The tissues were dehydrated in ethanol and chloroform and embedded in paraffin wax for subsequent histological analysis. This procedure was performed both in freshly isolated vessels and in some that had been used for functional investigation. Serial 3 μ m transverse sections were cut using a rotary microtome and mounted onto poly-L-lysine coated glass slides. Sections were oven dried overnight at 37°C. General morphology was established using a combination of the stains haematoxylin and eosin, which show nuclei and cellular tissue components, respectively. Smooth muscle and endothelial cells were detected using mouse anti α -smooth muscle actin IgG (1:50 dilution) and biotinylated ulex europaeus agglutinin (1:100 dilution), respectively. α -sma IgG (but not ulex which was already biotinylated) was incubated with a goat anti-mouse biotinylated secondary antibody. Both smooth muscle and endothelium were detected using a streptavidin-biotin-peroxidase kit, followed by incubation with diaminobenzidine. Positive signals appeared brown. Negative controls were obtained by omitting the primary antibody (for detailed methodology refer to Chapter 2).

5.3.3. *Functional Investigations*

Human subcutaneous resistance vessels, approximately 2mm long, were mounted as ring preparations on two 40 μ m intraluminal wires in an isometric small vessel myograph, containing PSS, maintained at 37°C and equilibrated with 95% O₂/5% CO₂, for measurement of isometric force. Following a 30min equilibration period, the vessels were subject to a standard normalisation procedure (detailed in Chapter 2) and set to their optimum resting level ($0.9L_{100}$, where L_{100} is the internal circumference the vessels would have when relaxed and subjected to a pressure of 13.3kPa). After a further 30min equilibration, the vessels were then subjected to a standard start procedure, which consisted of five consecutive stimulations of 3min each, followed by a 5min washout period. The first, second and fifth contractions were produced using high (125mM) potassium solution (KPSS; made by equimolar substitution of KCl for NaCl in PSS) containing 10 μ M noradrenaline (NA). The third was obtained with NA (10 μ M) alone and the forth with KPSS alone. The functional integrity of the endothelium was assessed by adding ACh (0.1-10 μ M) to vessels submaximally contracted with NA (3 μ M). In some vessels, the endothelium was removed by rubbing the lumen with a human hair (Chapter 2).

5.3.3.1. Functional Characteristics of Small and Large Resistance Arteries and Veins.

Cumulative concentration-response curves were obtained in all three vessel types using the receptor-dependent agonists NA (10^{-9} - 3×10^{-5} M) and ET-1 (10^{-11} - 3×10^{-7} M) and the receptor-independent vasoconstrictor K⁺ (10-125mM). Cumulative concentration-response curves were obtained for the receptor-dependent, endothelium-dependent

vasodilators ACh (10^{-9} - 3×10^{-5} M) and BK (10^{-10} - 3×10^{-6} M), the receptor-independent, endothelium-dependent dilator A23187 (10^{-9} - 3×10^{-6} M) and the endothelium-independent vasodilator, SIN-1 (10^{-9} - 3×10^{-5} M). Responses to vasodilators were obtained following precontraction of the vessel with a submaximal concentration ($3 \mu\text{M}$) of NA.

5.3.3.2. Functional Identification of Mediators of Endothelium-Dependent Relaxation in Small Resistance Arteries

Each artery was precontracted with a submaximal concentration of NA ($3 \mu\text{M}$) and a cumulative concentration-response curve was obtained in response to ACh (10^{-9} - 3×10^{-5} M). On completion of the curve the contents of the bath were changed and the arteries were bathed in fresh PSS. In order to determine the relative contributions of PGI_2 , NO and EDHF as mediators of endothelium-dependent relaxation, the procedure was repeated following incubation with either:- (a) indomethacin ($10 \mu\text{M}$ for 45min), (b) N^G -nitro-L-arginine (L-NOARG; $100 \mu\text{M}$ for 45min) or (c) L-NOARG ($100 \mu\text{M}$ for 45min) plus charybdotoxin (ChTx; 50nM for 10min) and apamin (30nM for 10min). Most arteries were exposed to only one antagonist but, in order to obtain three groups of six vessels, two of those initially incubated with indomethacin were subsequently incubated with L-NOARG (in the absence of indomethacin) before production of a further concentration-response curve to ACh. The effect of L-NOARG on ACh-mediated relaxation in these two arteries was not different from the effect on arteries exposed only to L-NOARG.

Three of the arteries incubated with the combination of L-NOARG, ChTx and apamin, were exposed to a single concentration (100 μ M) of the endothelium-independent vasodilator 3-morpholinosydnonimine (SIN-1) once the cumulative concentration-response curve to ACh had been completed.

5.3.4. *Drugs*

Reagents for the preparation of PSS were obtained from BDH Laboratory Supplies, Poole, Dorset, UK. All drugs were purchased from Sigma, Poole, Dorset, UK. Acetylcholine chloride and noradrenaline bitartrate were dissolved in distilled water (10⁻³M stock); indomethacin (10⁻³M stock) in 1.5x10⁻³M Na₂CO₃ (final bath concentration of Na₂CO₃ did not exceed 0.015mM) and apamin (10⁻⁵M stock) in 0.05M acetic acid (final bath concentration of acetic acid did not exceed 0.15mM). L-NOARG (10⁻²M stock) was dissolved in 0.1N hydrochloric acid. Charybdotoxin (10⁻⁶M stock) was dissolved in a Tris buffer (10mM, pH 7.5) containing 0.1% BSA, 100mM NaCl and 1mM EDTA (final bath concentrations of NaCl and EDTA did not exceed 5mM and 0.05mM, respectively). 0.01% BSA was added to the myograph chamber before applying the toxins. All drugs were stored as stock solutions at -20°C and thawed as required; subsequent dilutions were made in distilled water and any residual solution was discarded at the end of the experiment. The concentration given is the final molar concentration in the bath.

5.3.5. *Statistics*

All values are presented as mean \pm standard error mean (s.e.mean) from n experiments (where n represents the number of subjects). Contractions are given as mN/ mm and the

relaxation to ACh is expressed as a percentage reversal of the initial NA-induced precontraction. Sensitivities to vasoconstrictors and to ACh are expressed as the negative log of the effective concentration (M) of the drug required to produce 50% of the maximum effect (pD₂ and -logIC₅₀, respectively). The sensitivities were calculated from each concentration-response curve by fitting the data to the Hill equation using curve fitting software (Fig. P, Biosoft, Cambridge). Comparison of maximum response and sensitivities between small and large resistance arteries and veins were made using Student's unpaired t-test. Student's paired t-test was used in the comparison of maximum response and sensitivity before and after incubation with the inhibitors. Significance was assumed if $P < 0.05$.

5.4. Results

Large and small resistance arteries, and small veins, isolated from the biopsies of subcutaneous fat were distinguished using physical differences as described in the methods section.

5.4.1. Morphology

Haematoxylin and eosin staining demonstrated that small resistance arteries had a clearly defined media and adventitia (Figure 5.1A(ii)). Immunohistochemistry showed that the media contained mainly smooth muscle cells (Figure 5.1A(iii)) with a monolayer of endothelial cells lining the internal elastic lamina (Figure 5.1A(iv)). In contrast, veins failed to hold their shape and distinction between media and adventitia was less obvious following staining with haematoxylin and eosin (Figure 5.1B(ii)). However, immunohistochemistry demonstrated that the media stained strongly for α -

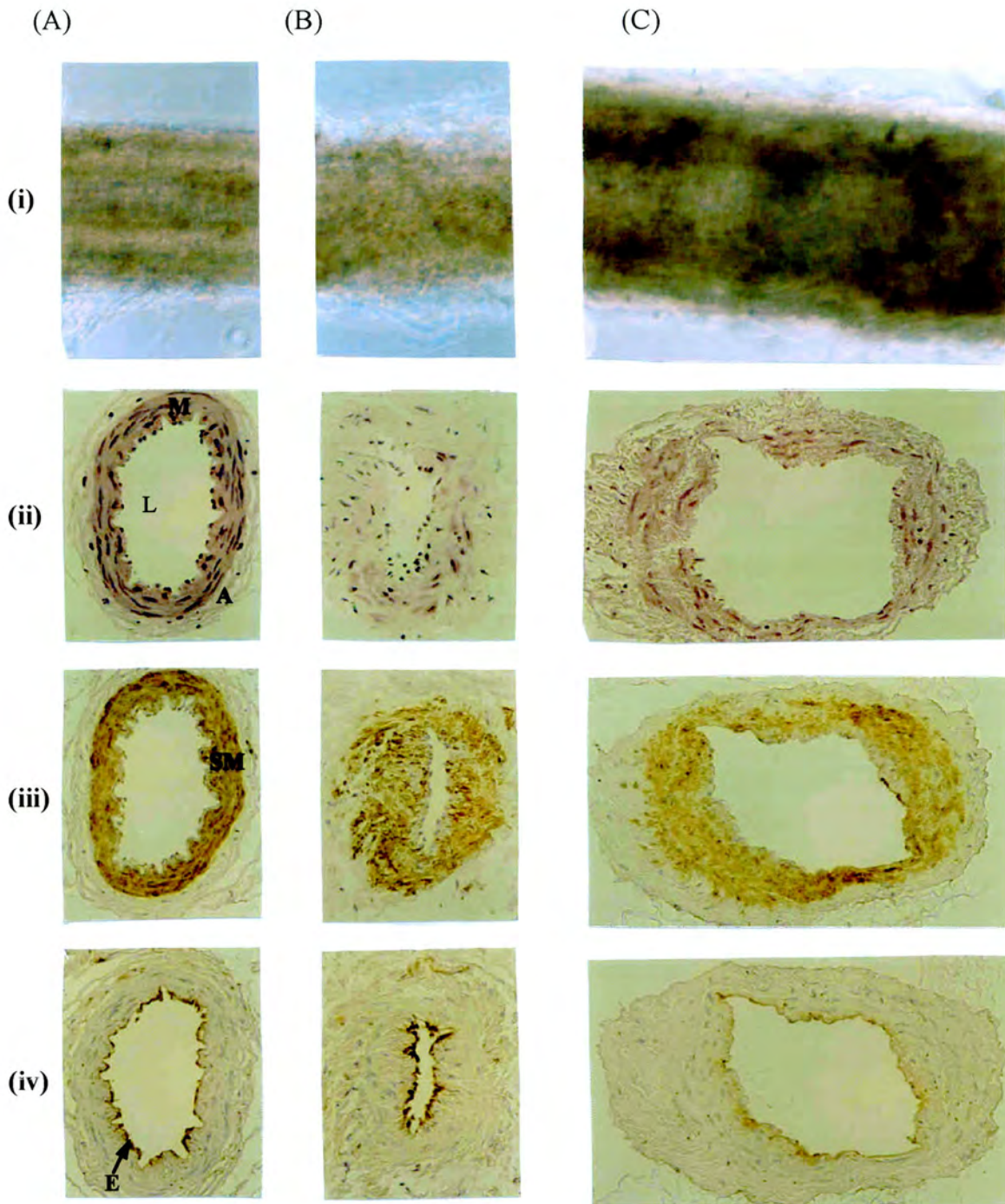


Figure 5.1. Physical and morphological differences between small resistance arteries (A), veins (B) and large resistance arteries (C) isolated from human subcutaneous fat biopsies. (i) light microscopy showing differences in physical characteristics between the three vessel types. (ii) haematoxylin and eosin staining showing media (M), lumen (L) and adventitia (A). (iii) and (iv) immunohistochemical localisation of smooth muscle (SM) and endothelium (E) using monoclonal antibodies against α -smooth muscle actin and ulex europaeus agglutinin, respectively. Magnification x200.

sma (Figure 5.1B(iii)) and the ulex antibody identified a clear endothelium lining the lumen (Figure 5.1B(iv)). The large resistance arteries had a clearly defined media and adventitia when stained with haematoxylin and eosin (Figure 5.1C(ii)). Like the small resistance arteries and veins, the media comprised mainly of smooth muscle cells (Figure 5.1C(iii)) and an endothelial layer was evident on the luminal surface (Figure 5.1C(iv)).

5.4.2. *Normalisation*

Normalisation, a procedure which determines the internal circumference which the vessel would have if relaxed and under a transmural pressure of 100mmHg (13.3kPa), is performed by distending the vessel stepwise until the effective transmural pressure exceeds 100mmHg (see Chapter 2 for details). Normalisation of small resistance arteries was uncomplicated with the effective transmural pressure exceeding 13.3kPa after three to four stretches. In contrast, large resistance arteries and veins did not maintain stretch as well as the small resistance arteries and typically required in excess of 6 stretches to fully complete the normalisation procedure. The internal diameter of large resistance arteries ($519 \pm 72 \mu\text{m}$; $n=7$) was significantly greater ($P<0.005$) than that of the small resistance arteries ($168 \pm 18 \mu\text{m}$; $n=7$) when stretched to an effective pressure of 13.3kPa. Although veins were chosen which appeared similar in size to the small resistance arteries, they were found to have an internal diameter ($410 \pm 93 \mu\text{m}$; $n=5$) similar ($P=0.37$) to the large resistance arteries when an effective pressure of 13.3kPa was exerted.

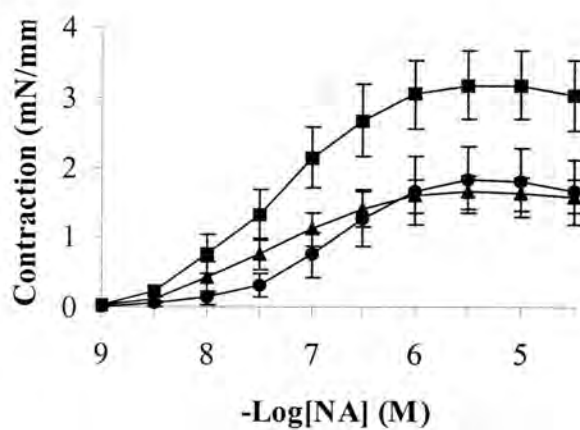
5.4.3. *Vascular Function*

5.4.3.1. Functional Characteristics of Large and Small Resistance Arteries and Veins

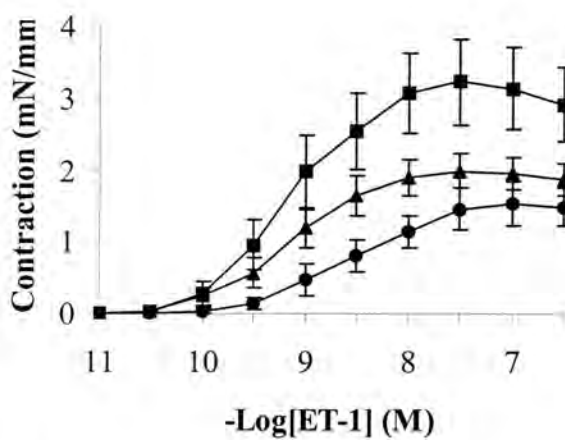
All three types of vessel produced concentration-dependent contractions in response to the vasoconstrictors NA, ET-1 and KPSS (Figure 5.2). The maximum contractile response to NA tended ($P=0.07$) to be greater in the large than in the small resistance arteries and veins, although this difference only reached significance ($P=0.03$) when compared with veins. Furthermore, this difference was still apparent when the contractions were expressed as a percentage of the maximum response to NA-K to control for variations in vessel size (Table 5.1.). Sensitivity to NA was significantly ($P=0.01$) greater in large (pD_2 ; 7.34 ± 0.18 ; $n=7$), compared with small (pD_2 ; 6.74 ± 0.11 ; $n=7$) resistance arteries, but was similar ($P=0.80$) to the sensitivity exhibited by veins (pD_2 ; 7.40 ± 0.14 ; $n=5$).

The contraction produced by ET-1 (Figure 5.2b) was greater ($P=0.02$) in large compared with small resistance arteries and tended ($P=0.10$) to be stronger than the response produced in veins (Table 5.1). However, these differences were lost when the results were expressed as a percentage of the maximum response to NA-K, to control for variations in vessel size (Table 5.1). Large vessels also tended ($P=0.09$) to be more sensitive (pD_2 , 9.05 ± 0.16 , $n=7$) to ET-1 than small resistance arteries (pD_2 , 8.61 ± 0.17 , $n=7$) but veins showed a similar sensitivity (pD_2 , 9.19 ± 0.20 , $n=5$; $P=0.60$).

(a) Noradrenaline



(b) Endothelin-1



(c) Potassium

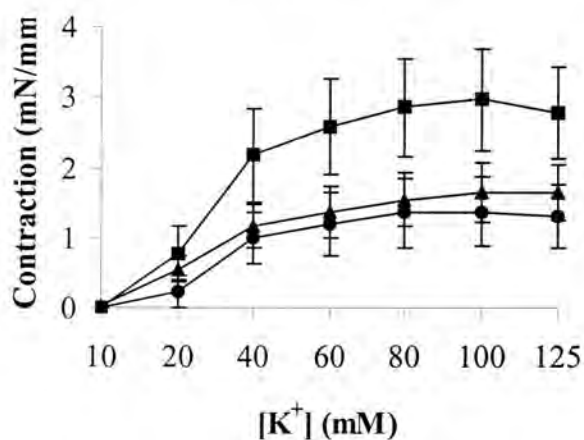


Figure 5.2. Concentration-response curves to (a) noradrenaline, (b) endothelin-1 and (c) K^+ in small resistance arteries (●, $n=7$), veins (▲, $n=5$) and large resistance arteries (■, $n=7$) isolated from human subcutaneous fat biopsies. Each point represents mean \pm s.e.mean.

Table 5.1. Maximum contraction (Emax) of small and large resistance arteries and veins to noradrenaline (NA), endothelin-1 (ET-1) and KPSS.

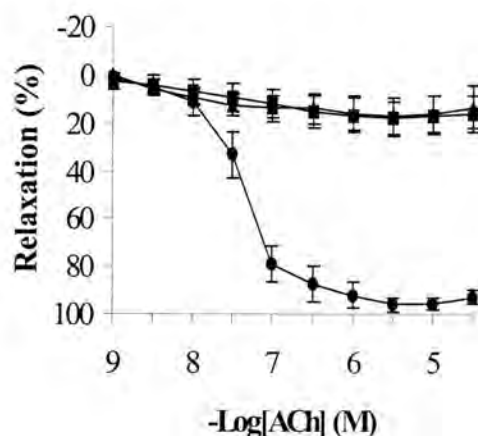
	Emax (mN/mm)			Emax (%NA-K)		
	NA	ET-1	KPSS	NA	ET-1	KPSS
SRA (n=7)	1.83±0.49	1.57±0.30*	1.39±0.49	97.99±7.06	92.98±10.96	69.70±13.31
LRA (n=7)	3.21±0.49	3.35±0.60	2.99±0.72	107.49±1.92	111.90±8.03	96.02±16.77
V (n=5)	1.65±0.24*	2.00±0.24	1.67±0.41	96.12±3.41*	122.08±16.57	94.57±14.78

Results are shown as mean \pm s.e.mean and are expressed as force per unit length of tissue (mN/mm) and as a percentage of the maximum response to NA-K, to control for variations in vessel size. * $P<0.05$ when compared with large resistance artery. SRA, small resistance artery; LRA, large resistance artery; V, vein.

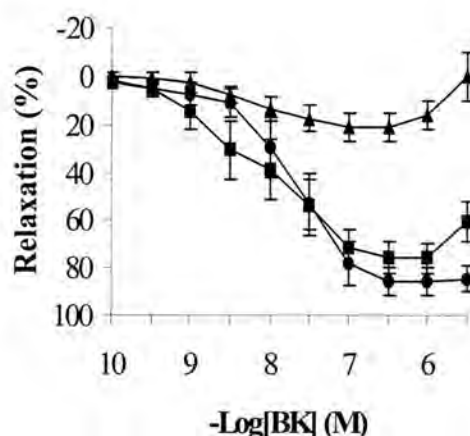
As with NA and ET-1, the E_{max} to KPSS (Figure 5.2c) produced by large resistance arteries tended to be greater than that produced by small resistance arteries ($P=0.09$) and veins ($P=0.19$; Table 5.1). However, the sensitivity (pD_2) to this constrictor in large resistance arteries (1.53 ± 0.04 , $n=7$) was similar to that in small resistance arteries (1.51 ± 0.04 , $n=7$; $P=0.65$) and veins (1.58 ± 0.06 , $n=5$; $P=0.50$).

Small resistance arteries relaxed in a concentration-dependent manner in response to ACh (Figure 5.3a) (E_{max} , $96.72 \pm 2.68\%$, $-\log IC_{50}$, 7.34 ± 0.11 , $n=7$). However, exposure to this agonist failed to elicit an active relaxation in large resistance arteries (E_{max} , $17.43 \pm 7.95\%$; $n=7$) or veins (E_{max} , $18.20 \pm 7.28\%$; $n=5$) (Figure 5.3a). BK-mediated relaxation was similar in small (E_{max} , $86.92 \pm 5.31\%$; $-\log IC_{50}$, 7.63 ± 0.14 ; $n=7$) and large (E_{max} , $78.13 \pm 5.93\%$, $P=0.29$; $-\log IC_{50}$, 7.96 ± 0.27 , $P=0.31$, $n=7$) resistance arteries. In contrast, veins failed to relax in response to BK (E_{max} , $20.56 \pm 6.68\%$, $n=5$) (Figure 5.3b). Relaxation to A23187 (Figure 5.3c) was similar in large (E_{max} , $67.04 \pm 8.36\%$; $-\log IC_{50}$, 6.63 ± 0.20 ; $n=7$) and small (E_{max} , $76.00 \pm 11.52\%$, $P=0.54$; $-\log IC_{50}$, 6.76 ± 0.23 , $P=0.66$; $n=7$) resistance arteries, but only produced relaxation in two out of five veins (E_{max} 52.89% and 91.18%). All three vessel types relaxed in response to SIN-1 (Figure 5.3d). The magnitude (E_{max} , $99.19 \pm 3.86\%$, $n=7$) and sensitivity ($-\log IC_{50}$, 5.92 ± 0.21 , $n=7$) of this response in large resistance arteries were similar to that produced by small resistance arteries (E_{max} , 98.33 ± 3.44 , $P=0.87$; $-\log IC_{50}$, 6.21 ± 0.14 , $P=0.27$) and veins (E_{max} , 106.22 ± 18.65 , $P=0.67$; $-\log IC_{50}$, 5.52 ± 0.11 , $P=0.17$).

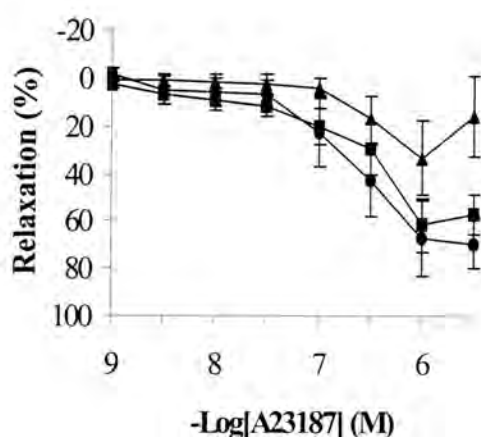
(a) Acetylcholine



(b) Bradykinin



(c) A23187



(d) SIN-1

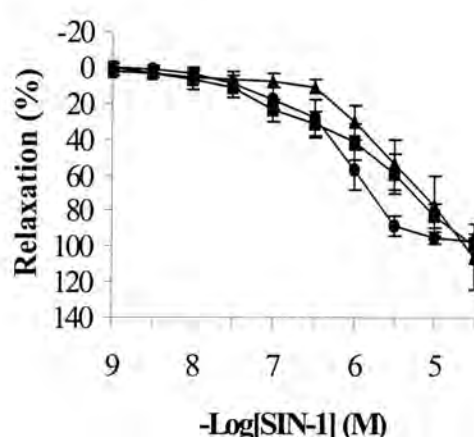


Figure 5.3. Concentration-response curves to the vasodilators (a) acetylcholine, (b) bradykinin, (c) A23187 and (d) 3'-morpholinodisodium (SIN-1) in small resistance arteries (●, n=7), veins (▲, n=5) and large resistance arteries (■, n=7) isolated from human subcutaneous fat biopsies. Each point represents mean \pm s.e.mean.

Based on the criteria for distinguishing the different vessels isolated from the biopsies, vessels identified as large resistance arteries or veins were discarded and subsequent experiments with the inhibitors were only performed in vessels identified as small resistance arteries.

5.4.3.2. Functional Identification of Mediators of Endothelium-Dependent Relaxation of Human Small Resistance Arteries

Human subcutaneous small resistance arteries pre-contracted with a submaximal concentration of NA ($3\mu\text{M}$), relaxed in a concentration-dependent manner when exposed to the endothelium-dependent vasodilator ACh (10^{-9} - $3\times 10^{-5}\text{M}$). In each of the three groups of arteries, this produced approximately 90% relaxation in the absence of the inhibitors (see below) and was abolished by removal of the endothelium (Figure 5.4.). Addition of, and incubation with, the inhibitors did not increase the resting tone of any of the arteries. Furthermore, the amplitude of the response to the pre-contracting concentration ($3\mu\text{M}$) of NA was not increased following incubation with any of the inhibitors (Table 5.2.). Indeed, the trend was for the second pre-contraction to be smaller than the first, probably as a result of tachyphylaxis. This was most apparent with the arteries incubated with the combination of L-NOARG, ChTx and apamin, but did not reach significance.

5.4.3.2.1. *Effect of cyclooxygenase inhibition*

Following incubation with indomethacin ($10\mu\text{M}$ for 45min), the magnitude of ACh-induced endothelium-dependent relaxation was not significantly different from the

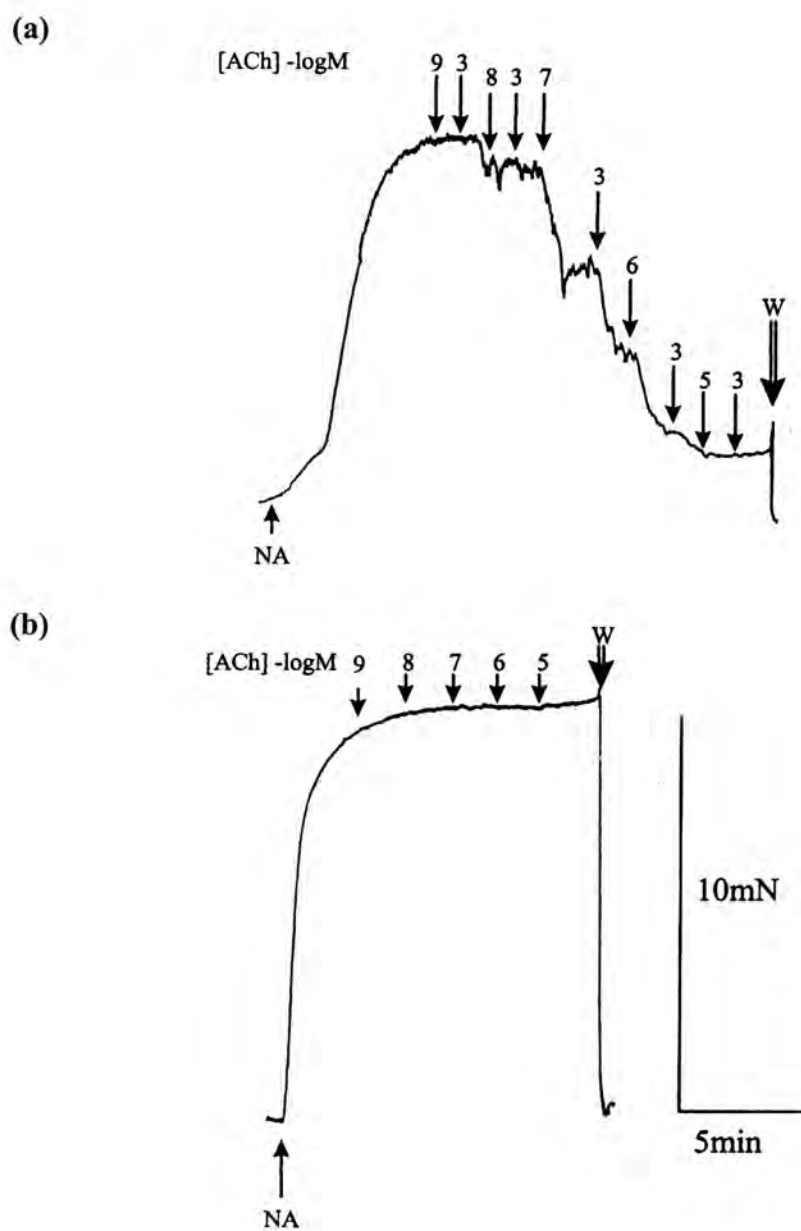


Figure 5.4. Trace demonstrating the effect of acetylcholine (ACh) in a human small resistance artery submaximally precontracted with noradrenaline (NA) before (a) and after (b) removal of the endothelium. W-washout.

Table 5.2. Magnitude of precontraction to NA (3 μ M), in human small resistance arteries, before and after incubation with either indomethacin (10 μ M for 45min), L-NOARG (100 μ M for 45min) or L-NOARG plus ChTx (50nM for 10min) and apamin (30nM for 10min).

	NA Precontraction (mN/mm)		
	Before	After	<i>P</i>
Indomethacin	2.16 \pm 0.40	2.02 \pm 0.40	0.12
L-NOARG	2.58 \pm 0.32	2.53 \pm 0.33	0.52
L-NOARG + ChTx + Apamin	1.91 \pm 0.44	1.61 \pm 0.38	0.053

Values are expressed as force per unit length of tissues (mN/mm) and are shown as mean \pm s.e.mean, n=6 for each group.

control (maximum relaxation (%)) 90.80 ± 4.69 vs 97.56 ± 1.83 , $P=0.18$, for control vs indomethacin, $n=6$). The sensitivity of the response to ACh following exposure to indomethacin was not altered compared with controls ($-\log IC_{50}$ values 7.23 ± 0.25 vs 7.24 ± 0.20 , $P=0.96$, for control vs indomethacin, $n=6$. Figure 5.5a.).

5.4.3.2.2. *Effect of NOS inhibition.*

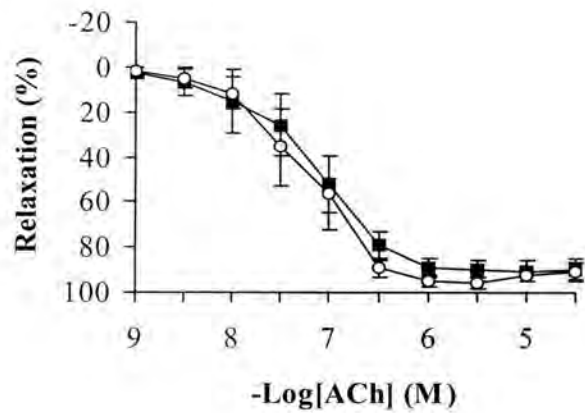
Following exposure to L-NOARG, ACh-evoked relaxation was significantly reduced, but not completely abolished (maximum relaxation (%)) 91.55 ± 3.95 vs 61.68 ± 3.38 , $P<0.0001$), with a corresponding rightward shift of the ACh concentration-response curve ($-\log IC_{50}$ values 7.19 ± 0.13 vs 6.41 ± 0.10 , $P<0.005$, for control vs L-NOARG, $n=6$. Figure 5.5b.).

5.4.3.2.3. *Effect of potassium channel blockade*

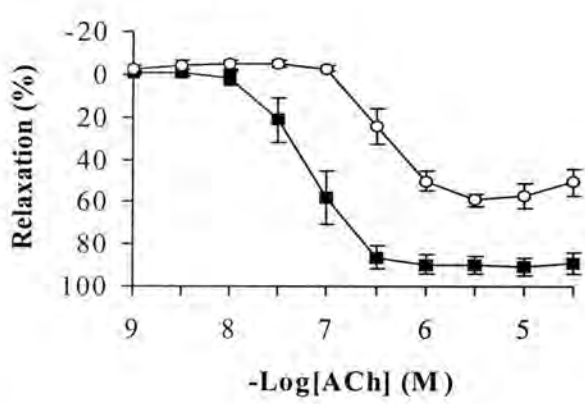
Incubation of arteries with a combination of L-NOARG plus the potassium channel blockers ChTx (50nM for 10min) and apamin (30nM for 10min) virtually abolished the L-NOARG resistant component of ACh-induced relaxation (maximum relaxation (%)) 92.59 ± 3.65 vs 15.2 ± 10.5 , $P<0.002$, for control vs ChTx & apamin, $n=6$. Figure 5.5c.). The sensitivity could not be calculated following incubation with the K^+ channel blockers.

Addition of the endothelium-independent dilator, SIN-1 (100 μ M) caused complete relaxation ($127.6 \pm 13.0\%$; $n=3$) of vessels incubated with the combination of L-NOARG, ChTx and apamin.

(a) Indomethacin



(b) L-NOARG



(c) L-NOARG + Charybdotoxin + Apamin

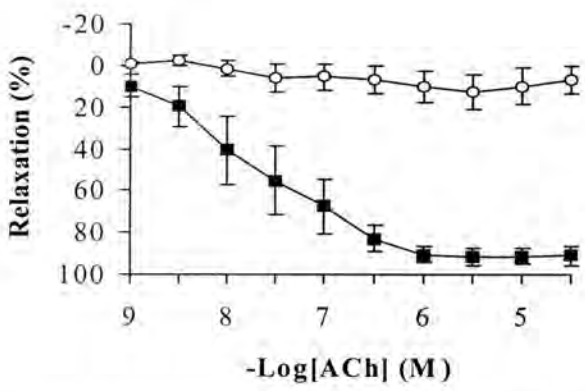


Figure 5.5. Concentration-response curves to acetylcholine in isolated human resistance arteries before (■) and after (○) incubation with either (a) indomethacin (10μM for 45min), (b) L-NOARG (100μM for 45min) or (c) L-NOARG plus ChTx (50nM for 10min) and apamin (30nM for 10min). Each point represents mean ± s.e.mean, n=6 for each group.

5.5. Discussion

This study has demonstrated the existence of three functionally distinct vessels obtained from biopsies of human subcutaneous fat and secondly that ACh-induced endothelium-dependent relaxation of noradrenaline contracted human subcutaneous resistance arteries is mediated by both NO-dependent and independent pathways.

5.5.1. Vessel Identification

Gluteal subcutaneous fat biopsies provide a convenient source of resistance arteries for investigating the vascular consequences of a variety of conditions, including hypertension (Deng *et al.*, 1995) and pre-eclampsia (Aalkjaer *et al.*, 1984). However, this investigation has demonstrated the existence of three functionally distinct vessels isolated from biopsies of human subcutaneous fat: small and large resistance arteries and veins. The most apparent difference was the inability of large resistance arteries to relax in response to the endothelium-dependent vasodilator ACh.

ACh-induced vasodilatation results from the release of relaxing factors following stimulation of muscarinic receptors on the endothelium. The failure of ACh to relax large resistance arteries has been reported previously (Angus *et al.*, 1992) and could be due to (i) damage to the endothelium, (ii) reduced smooth muscle responsiveness to relaxant factors or (iii) lack of endothelial muscarinic receptors. Endothelial damage as a result of vessel isolation is unlikely to account for the lack of response of large resistance arteries to ACh as immunohistochemistry clearly demonstrated an intact endothelial cell layer, and responses to other endothelium-dependent vasodilators (BK and A23187) were similar to those responses obtained in small resistance arteries.

Likewise, the lack of response to ACh in large resistance arteries is unlikely to be due to reduced responsiveness of vascular smooth muscle cells to relaxing factors, as endothelium-independent responses to SIN-1 were comparable with those in small resistance arteries, indicating that the smooth muscle was able to relax in response to NO. Therefore, the lack of response to ACh in large resistance arteries is most likely due to an absence of muscarinic receptors on the endothelial cells. This observation reinforces the demonstration of receptor heterogeneity in the endothelium (Vanhoutte, 1989) and is consistent with those reported by Angus *et al* (1992) who demonstrated that large subcutaneous resistance arteries which failed to relax to ACh relaxed when exposed to substance P.

In order to allow comparison with small and large resistance arteries, the functional responses of subcutaneous veins were also investigated. The lack of responsiveness to endothelium-dependent vasodilators (ACh and BK) is consistent with previous observations in large systemic veins (Vanhoutte & Miller, 1985; DeMey & Vanhoutte, 1982; Luscher *et al.*, 1988). Immunohistochemistry indicated that the veins had an intact endothelium, suggesting that the lack of responses to these vasodilators may be due to the lack of necessary receptors on the endothelial cells. However, the variable response to A23187 contrasts with some systemic veins which, despite responding poorly to ACh, relax significantly in response to A23187 (Luscher *et al.*, 1988). There is evidence that this may be due to anatomical variation in this response and this may account for the heterogeneity of the response to A23187 in the veins used in the present study. The failure to relax to endothelium-dependent vasodilators, however, was not due to reduced vascular smooth muscle sensitivity as exposure to SIN-1 caused complete

relaxation of human subcutaneous veins, comparable with that of small resistance arteries.

The three vessel types all contracted in a concentration-dependent manner in response to NA, ET-1 and KPSS. The increased maximum response to these agonists in large resistance arteries compared with small resistance arteries and veins is most likely the result of size differences, as responses were similar if the results were expressed as a percentage of the response to NA-K, to control for variations in vessel size. The observed increase in sensitivity to NA and ET-1, but not to KPSS, in large resistance arteries and veins compared with small resistance arteries may suggest heterogeneity in NA/ET receptor populations. Alternatively, it is possible that the inhibitory role of the endothelium on contractile response that has been reported in small resistance arteries, is absent from large resistance arteries and veins.

5.5.2. *Role of the L-arginine/NO pathway in ACh relaxation*

Since the discovery of NO as a major endothelium-derived relaxing factor (Palmer *et al.*, 1987), structural analogues of its precursor, L-arginine (Palmer *et al.*, 1988), have been used to abolish endothelium dependent relaxation in a variety of arteries (Rees *et al.*, 1989; Rees *et al.*, 1990; Mulsch & Busse, 1990). In some vessels, particularly resistance arteries, endothelium-dependent relaxation may remain partially or wholly insensitive to NOS inhibition (Shimokawa *et al.*, 1996; Urakami- Harasawa *et al.*, 1997). Our finding that ACh-evoked endothelium-dependent relaxation is only partially inhibited following NOS inhibition is in accordance with previous *in vitro* data on human small arteries (Woolfson & Poston, 1990; Deng *et al.*, 1995; Hillier *et al.*, 1998).

Similar results have been obtained with other endothelium-dependent agonists such as BK and substance P in human arteries from various anatomical (omental, pial, gastroepiploic) locations (Petersson *et al.*, 1995; Ohlmann *et al.*, 1997; Urakami-Harasawa *et al.*, 1997). The failure of L-NOARG to completely block ACh-mediated relaxation is unlikely to be related to incomplete inhibition of NO synthase as a lower concentration (3×10^{-5} M) abolished endothelium-dependent relaxation in the rat aorta, pulmonary and iliac arteries (Nagao *et al.*, 1992). Furthermore, the incomplete inhibition of ACh-mediated relaxation in the rabbit renal artery was not overcome by increasing the concentration of L-NOARG (100-300 μ M; Brandes *et al.*, 1997) or by the combined application of two different L-arginine analogues (Plane & Garland, 1996; Plane *et al.*, 1997). These results suggest, therefore, that a NO-independent mechanism is responsible for the relaxation that persists in the presence of L-NOARG.

5.5.3. *Role for prostanoids in ACh relaxation*

Cyclo-oxygenase activity has been demonstrated in both the endothelium and vascular smooth muscle (Moncada, 1982), implicating endothelium-derived prostaglandins as mediators of endothelium-dependent relaxation. Indeed, PGI₂ contributes to endothelium-dependent relaxation in the rabbit mesenteric, coeliac and coronary artery beds (Forstermann *et al.*, 1986; Lamantagne *et al.*, 1992), with PGE₂ assuming greater significance in the rabbit extra-pulmonary artery (Forstermann *et al.*, 1986). Bradykinin (BK)-mediated relaxation of human omental resistance arteries may also be partially mediated by prostaglandins (Ohlmann *et al.*, 1997). In the present study, the inability of the cyclooxygenase inhibitor indomethacin to inhibit ACh-mediated relaxation suggests that vasodilator prostanoids do not contribute to this response in human subcutaneous

resistance arteries. This is consistent with results obtained in similar vessels (Deng *et al.*, 1995; Hillier *et al.*, 1998) as well as in human gastroepiploic resistance arteries (Urakami-Harasawa *et al.*, 1997), and also reflects the inability of indomethacin to inhibit substance P-mediated relaxation of human omental vessels (Wallerstedt & Bodelsson, 1997). It is possible, however, that the effects of cyclooxygenase inhibition only become apparent when production of NO is also blocked. In human subcutaneous (Woolfson & Poston, 1990) and omental (Ohlmann *et al.*, 1997) resistance arteries a small, indomethacin-sensitive component of relaxation (to ACh and BK, respectively) was evident only in the presence of L-arginine analogues (L-Nitroarginine methyl ester (L-NAME); L-N^G-monomethyl arginine (L-NMMA)). In contrast, however, indomethacin had no effect on BK-mediated relaxation either in the absence or presence of L-NAME in human subcutaneous resistance arteries (Hillier *et al.*, 1998). Interestingly, in the present study the ACh-mediated relaxation tended to be greater in vessels incubated with indomethacin, although this difference did not reach statistical significance. This result is similar to that reported recently by Hillier *et al.* (1998) and may suggest that indomethacin inhibits the action of contractile prostanoids, thus enabling ACh to have a greater relaxant effect.

5.5.4. *Role for EDHF in ACh relaxation*

Previous investigations have demonstrated that an NO/PG-independent component of ACh-induced relaxation is mediated by EDHF (Nakashima *et al.*, 1993; Urakami-Harasawa *et al.*, 1997; Wallerstedt & Bodelsson, 1997). In the current investigation, the role of EDHF in human subcutaneous resistance arteries was assessed using a combination of the inhibitors previously applied to a variety of vessels including rat

hepatic (Zygmunt & Högestätt, 1996), guinea pig basilar and human pial (Petersson *et al.*, 1997) arteries. This combines the large (BK_{Ca}) and small (SK_{Ca}) calcium-dependent K^+ channel inhibitors, ChTx and apamin, in the continuing presence of a NO synthase inhibitor (Zygmunt & Högestätt, 1996).

The results from this study demonstrate that the combination of ChTx and apamin virtually abolished the NO-independent component of ACh-induced relaxation. This was not due to a toxic effect on the vascular wall for, as in a previous study (Deng *et al.*, 1995), the arteries retained the ability to relax in response to an endothelium-independent dilator. These results correspond with those obtained in a variety of isolated vessels, including guinea pig basilar (Petersson *et al.*, 1997), and rat mesenteric (Plane *et al.*, 1997) and hepatic, arteries (Zygmunt & Högestätt, 1996), which indicate that the ChTx/apamin-sensitive, NO-independent component of ACh-evoked relaxation is mediated by EDHF. In rat mesenteric arteries, the NO-independent component of ACh-mediated relaxation is accompanied by smooth muscle cell hyperpolarization (Plane & Garland, 1996). This response is abolished by the combination of ChTx and apamin (Zygmunt & Högestätt, 1996), probably by inhibition of BK_{Ca} and SK_{Ca} on the endothelium (Doughty *et al.*, 1999).

Several studies have investigated the relative contribution of relaxing factors to the endothelium-dependent relaxation produced by bradykinin (Nakashima *et al.*, 1993; Ohlmann *et al.*, 1997; Urakami-Harasawa *et al.*, 1997; Pascoal & Umans, 1996) and substance P (Petersson *et al.*, 1995; Wallerstedt & Bodelsson, 1997) in human arteries. Exposure of human omental microvessels and gastroepiploic arteries to ChTx and/or

apamin had little or no effect on NO-independent relaxation to bradykinin (Pascoal & Umans, 1996; Ohlmann *et al.*, 1997; Urakami-Harasawa *et al.*, 1997). These results contrast with our findings in which NO-resistant relaxation to ACh was almost abolished by ChTx and apamin. There are several possible explanations for these differences. (i) the relative contribution of voltage-dependent and -independent mechanisms to endothelium-dependent relaxation may be influenced both by the contractile and relaxant agonists used (Plane *et al.*, 1992; Plane & Garland, 1996). Many previous studies which investigated the role of EDHF in human arteries have used different contractile agonists from that used in the present study (including arginine vasopressin (AVP; Pascoal & Umans, 1996), the thromboxane A₂ mimetic U46619 (Urakami-Harasawa *et al.*, 1997) and prostaglandin F_{2α} (PGF_{2α}; Ohlmann *et al.*, 1997). This may be significant as ACh-induced relaxation of rat mesenteric arteries is associated with hyperpolarisation following contraction with NA, but not following contraction with U46619 (Plane & Garland, 1996). Furthermore, human omental vessels pre-contracted with AVP relaxed in response to ACh (Pascoal & Umans, 1996) whereas those pre-contracted with U46619 did not (Wallerstedt & Bodelsson, 1997). (ii) The choice of dilator may be important as NO and EDHF alone were implicated in substance P-mediated relaxation of these arteries (Wallerstedt & Bodelsson, 1997) whilst prostanoids also contributed to BK-mediated relaxation (Ohlmann *et al.*, 1997). (iii) The effector mechanisms for EDHF may vary between vessels as a result of heterogeneous distribution of K⁺ channel subtypes as demonstrated in large and small vessels of the rat pulmonary tree (Albarwani *et al.*, 1995) and rabbit ear (Berman & Griffith, 1997).

The identity of EDHF remains elusive and it is possible that several different factors account for its activity: cytochrome P450 epoxygenic acids (Hecker *et al.*, 1994), endocannabinoids (Randall *et al.*, 1996) and myoendothelial gap junctions (Chaytor *et al.*, 1998). It has recently been proposed that EDHF is potassium released from ClTx and apamin sensitive potassium channels on the vascular endothelium which then acts on inwardly rectifying potassium channels and Na⁺/K⁺-ATPases on the smooth muscle cells to induce hyperpolarization (Edwards *et al.*, 1998). However, subsequent studies have challenged the identification of K⁺ as EDHF by demonstrating differences in the characteristics of EDHF and K⁺-mediated in rat mesenteric (Doughty *et al.*, 2000; Lacy *et al.*, 2000), porcine coronary and guinea pig carotid arteries (Quignard *et al.*, 1999). Furthermore, continuing work in our laboratory (Hadoke *et al.*, 1999) has indicated an obligatory role for the endothelium in K⁺-mediated relaxation in human small resistance arteries. This suggests that K⁺ is not an EDHF in these arteries but may be mediated by a further endothelium-dependent factor or is dependent on myoendothelial gap junctions (Doughty *et al.*, 2000).

In conclusion, this investigation demonstrated heterogeneity of ACh-mediated, endothelium-dependent relaxation in human subcutaneous resistance arteries of different sizes. This suggests variation in endothelial cell receptor populations as resistance artery diameter increases and indicates the importance of confirming that arteries isolated from subcutaneous fat have similar internal diameters and are responsive to both ACh and BK. Furthermore, this study demonstrated an NO/PG-independent response to ACh in human subcutaneous small resistance arteries, which had characteristics consistent with EDHF-mediated relaxation, suggesting that EDHF

may play an important role in the endothelium-dependent control of resistance artery tone.

CHAPTER 6

EFFECT OF DIABETES MELLITUS ON

HUMAN MICROVASCULAR REACTIVITY

IN VITRO

6.1. Introduction

Diabetes mellitus is associated with accelerated atherosclerosis and increased prevalence of cardiovascular disease. Both macrovascular disease (resulting in myocardial infarction, stroke and atheroma) and microvascular disease (resulting in diabetic retinopathy, nephropathy and neuropathy) are more prevalent in the diabetic, than in the non-diabetic, populations and contribute significantly to the increased morbidity and mortality associated with this disease (Watkins *et al.*, 1987; Eurodiab IDDM Complications Study Group, 1994).

There is substantial evidence to indicate that the ability of the endothelium to mediate vascular relaxation is impaired in large conduit arteries from animal models of diabetes (Poston & Taylor, 1995) and in patients with both type 1 (Zenere *et al.*, 1995; Lakakis *et al.*, 1997; Clarkson *et al.*, 1996) and type 2 (Morris *et al.*, 1995; Enderle *et al.*, 1998) diabetes mellitus. Similarly, contractile function may also be altered in these vessels, although the nature of this alteration remains controversial. These abnormalities have serious consequences as impaired function of conduit arteries has been implicated in the increased risk of atheroma in patients with diabetes.

Although it has been suggested that the microvascular complications associated with increased morbidity and mortality in diabetes are also the result of vascular dysfunction (Jaap & Tooke, 1995; Tooke, 1996), *in vivo* studies of microvascular function in patients with diabetes are contradictory. Endothelium-dependent relaxation has been reported as either unaltered (Calver *et al.*, 1992; Smits *et al.*,

1993; Avogaro *et al.*, 1997; Cockcroft *et al.*, 1998) or attenuated (Johnstone *et al.*, 1993; O'Driscoll *et al.*, 1997; Ting *et al.*, 1996; Gazis *et al.*, 1998) in patients with diabetes. Consequently, the development, nature and causes of functional alterations remain unclear. It would appear unlikely that diabetes would produce the same pathophysiological changes in conduit and resistance arteries, as these are functionally distinct. Receptor populations vary in arteries of different anatomical origin (Cambridge *et al.*, 1995) and the factors that contribute to endothelium-dependent relaxation differ in large compared with small vessels. Endothelium-dependent relaxation is mediated almost exclusively by NO in conduit arteries (Vanhoutte, 1989), whereas the contribution of an endothelium-derived hyperpolarizing factor becomes increasingly significant as vessel size decreases (Shimokawa *et al.*, 1996).

The few studies of vascular function using resistance arteries isolated from either patients with type 1 or type 2 diabetes have produced conflicting results. Endothelium-dependent relaxation induced by acetylcholine has been reported to be reduced (McNally *et al.*, 1994) or unaltered (Malik *et al.*, 1999, Lawrence *et al.*, 1994) in human resistance arteries. The impact of diabetes on contractile function is also controversial, with contraction of human resistance arteries reported as being unchanged (Lawrence *et al.*, 1994) or attenuated (McNally, *et al.* 1994).

6.2. Aims

The aim of the present investigation was to assess the impact of type 1 and type 2 diabetes on resistance artery function by studying both contractile and relaxant responses in resistance arteries taken from patients with these conditions.

6.3. Methods

6.3.1. Subjects

Two different groups of patients were studied. All patients were recruited from the diabetic outpatient clinic at the Western General Hospital, Edinburgh. Healthy, age and sex-matched, non-diabetic controls were selected for comparison with each of the diabetic groups. All subjects provided informed written consent and the study protocol was approved by the Lothian Research Ethics Committee.

6.3.1.1. Type 1 Diabetes

Twelve patients (seven male, five female) with type 1 diabetes and twelve age- and sex-matched controls were recruited. Patients with type 1 diabetes were selected on the basis that they had no history of cardiovascular disease and were taking no major drugs other than insulin. Controls were recruited from the general population and had no history of cardiovascular disease or diabetes. Six patients and four controls were smokers, and three patients were also taking oral contraceptives. Biopsies from eight patients and six controls provided sufficient vessels to allow investigation of the influence of elevated glucose concentrations on contractile and relaxant function.

6.3.1.2. Type 2 Diabetes

Eight male patients with type 2 diabetes and 10 age and sex matched controls were recruited. Patients with type 2 diabetes were being treated by diet alone and had no history of cardiovascular complications. Controls were recruited from the general population and had no history of cardiovascular disease or diabetes. Two patients and four controls were smokers.

6.3.1.3. Patient Assessment

Each individual enrolled in the study was subjected to a physical examination and a variety of tests, performed by clinical staff in the Metabolic Unit (Western General Hospital, Edinburgh). Blood pressure was measured three times (sitting, left arm; sitting, right arm and standing, left arm) to obtain mean values for systolic and diastolic pressure. The presence of retinopathy was assessed using dilated pupil ophthalmoscopy, whilst neuropathy was tested by measuring vibration in the toe. The presence of microalbuminuria was assessed in patients by calculation of the albumin/creatinine ratio (ACR). The determination of blood glucose, HbA_{1c}, serum total cholesterol and triglycerides was done using routine laboratory methods.

6.3.2. *Analysis of Resistance Artery Function*

Sub-cutaneous gluteal resistance arteries were obtained from both groups of patients and their controls. A biopsy of skin and subcutaneous fat (approximately 2cm long x 1cm x 1cm) was taken from the gluteal region under local anaesthesia (5ml of 2% lignocaine hydrochloride; Astra, Hertfordshire, UK). Resistance arteries were dissected from the biopsy and mounted as ring preparations on two 40µm stainless

steel wires in a small vessel myograph, containing physiological salt solution maintained at 37°C and perfused continuously with 95% O₂; 5% CO₂, for measurement of isometric tension. The vessels were set to their optimum resting force and, following a 30min equilibration at their optimum resting setting, vessel viability was assessed using a standard start procedure (see Chapter 2 for details).

6.3.3. Protocol

Resistance arteries from the studies of type 1 and type 2 diabetes were subjected to the same protocol in order to assess the influence of disease on vascular function. Cumulative concentration-response curves were obtained using the receptor-dependent agonists noradrenaline (NA; 10⁻⁹-3x10⁻⁵M) and endothelin-1 (ET-1; 10⁻¹¹-3x10⁻⁷M) and the receptor-independent vasoconstrictor, potassium (K⁺; 10-125mM). Responses to vasodilators were obtained following contraction of the artery with a sub-maximal concentration of NA (3μM). Using this approach, cumulative concentration-response curves were obtained for the receptor-dependent, endothelium-dependent agonists acetylcholine (ACh; 10⁻⁹-3x10⁻⁵M) and bradykinin (BK; 10⁻¹⁰-3x10⁻⁶M), the receptor-independent, endothelium-dependent calcium ionophore, A23187 (10⁻⁹-3x10⁻⁶M) and the endothelium-independent vasodilator 3'-morpholinosydnonimine (SIN-1; 10⁻⁹-3x10⁻⁵M). These concentration-response curves were always obtained in the same order (NA, ACh, BK, A23187, SIN-1, K⁺ and ET-1) with a washout period of 15-30min between each curve.

The influence of exposure to elevated glucose concentrations on resistance artery function was assessed using a protocol based on experience gained using rat

mesenteric arteries (Chapter 4). Arteries from patients with Type 1 diabetes and matched controls were selected arbitrarily for incubation for 1hr in PSS containing either normal (5.5mM) or elevated (20mM) concentrations of glucose. The incubation medium was changed every 30min and, before continuation of the protocol, the arteries were contracted for 3min with NA-K to confirm viability. The vessels were then exposed to vasoconstrictors and vasodilators using the protocol described above. Appropriate glucose concentrations were maintained for the duration of the experiment. After each CCRC, the contents of the bath were replaced four times with fresh PSS, containing the appropriate concentrations of glucose, and a 20min washout period was allowed between each CCRC.

6.3.4. *Statistics*

All values are mean \pm s.e.mean for n experiments, where n represents the number of subjects. Contractile responses are expressed as force per unit length of tissues (mN/mm) and as a percentage of the maximum response to the final stimulation with NA-K (% NA-K). Relaxation is expressed as a percentage relaxation of the initial precontraction with NA. Sensitivity values were obtained by fitting the Hill equation to the data using curve fitting software (Fig P, Biosoft, Cambridge, UK). Sensitivities are expressed as the negative logarithm of the concentration of the agonist required to produce 50% of the maximum effect (pD_2 for vasoconstrictors, $-\log IC_{50}$ for vasodilators). Comparison of maximum responses and sensitivities was analysed using Students unpaired or paired t-test, as appropriate, and significance was assumed when $P < 0.05$.

6.3.5. *Drugs*

All salts were obtained from BDH (Poole, Dorset, U.K.). Noradrenaline hydrochloride, acetylcholine chloride, bradykinin acetate salt and A23187 free acid were obtained from Sigma (Poole, Dorset, U.K.). Endothelin-1 and 3'-morpholinosydnonimine were obtained from Alexis (Nottingham, U.K.). Stock solutions (10^{-3} M) were prepared by dilution with distilled water for all drugs except endothelin-1 which was initially dissolved in 50% methanol to give a 10^{-5} M stock solution and A23187 which was initially dissolved in ethanol to give 10^{-4} M stock solution. Subsequent dilutions were made in distilled water and final bath concentrations of methanol and ethanol did not exceed 1.5% and 3% v/v, respectively. Stock solutions were frozen as 1ml aliquots at -20°C and thawed as required. Any residual solution was discarded at the end of the experiment.

6.4. *Results*

6.4.1 *Type 1 Diabetes*

Type 1 diabetic patients were evenly matched for age, sex, blood pressure, serum cholesterol and serum triglycerides (Table 6.1). As expected, HbA1c and glucose were significantly higher in type 1 diabetic patients compared with controls (Table 6.1). With one exception, the patients with type 1 diabetes had no history of macrovascular or microvascular complications, nor any evidence of retinopathy or neuropathy. One patient had evidence of proliferative retinopathy, but no elevation of albumin/creatinine ratio. Urinary albumin/creatinine ratios for the type 1 diabetic patients ($1.11 \pm 0.26 \text{mg/mmol}$, $n=12$) were within the normal range ($0.0\text{--}3.5 \text{mg/mmol}$), indicating normal renal function. The gluteal resistance arteries

Table 6.1. Baseline characteristics of control subjects and patients with type 1 diabetes.

	CONTROL	Type 1	P
Age (yrs)	31.5±2.2	32.3±1.9	0.78
Duration type 1 Diabetes (yrs)	-	13.9±2.5	-
Blood Pressure (mmHg) Systolic	122.6±2.7 (10)	121.3±3.3	0.78
Diastolic	79.2±2.4 (10)	77.1±2.1	0.51
Glucose (mmol/l)	4.85±0.07 (11)	13.84±1.84 (10)	<0.0001
Glycosylated Hb (%)	5.48±0.11	9.38±0.35	<0.000001
Serum Cholesterol (mmol/l)	4.55±0.36 (11)	5.25±0.28	0.13
Serum Triacylglycerol (mmol/l)	1.17±0.19 (11)	1.72±0.38	0.22
Serum Creatinine (μmol/l)	75.6±3.6 (11)	68.8±2.4	0.12
Serum Albumin (g/l)	42.08±0.63	38.42±3.34	0.38
Urea (mmol/l)	3.98±0.34	4.23±0.19	0.52

Values are mean ± s.e.mean. All values are for 12 observations except when indicated otherwise by n values quoted in parentheses, (n). Hb, haemoglobin;

isolated from patients (mean internal diameter $224 \pm 22 \mu\text{m}$) and controls ($195 \pm 9 \mu\text{m}$) were similar in size ($P=0.24$).

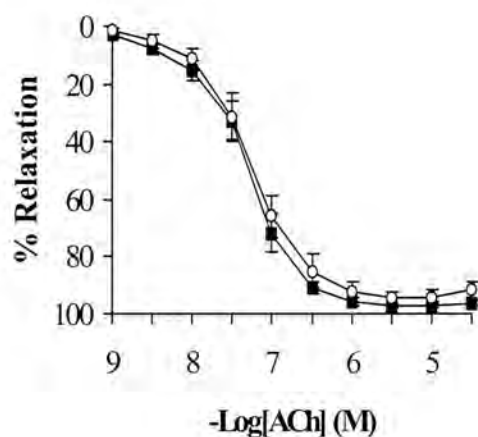
6.4.1.1. Responses to Vasodilators

The four vasodilators used in this study all evoked concentration-dependent relaxation in arteries from both patients and controls following contraction with a sub-maximal concentration of NA. ACh, BK and SIN-1 all produced approximately 100% relaxation in arteries isolated from patients and controls. A23187 produced a smaller (60-70%) relaxation than the other three vasodilators and did not achieve a definite maximum in the concentration range used (Figure 6.1.). The presence of type 1 diabetes had no apparent influence on vasodilator responsiveness as the size and sensitivity of the relaxations to ACh, BK, SIN-1 and A23187 were not different in arteries from patients compared with controls (Table 6.2). Responses to vasodilators in the three type 1 diabetic patients taking oral contraceptives were not different from those obtained with the rest of the group.

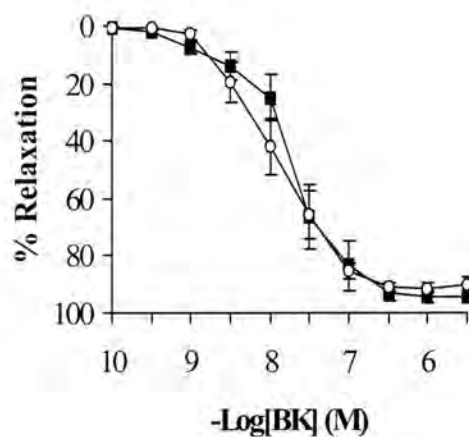
6.4.1.2. Responses to Vasoconstrictors

All the arteries produced concentration-dependent contractions in response to the vasoconstrictors ET-1, NA and K^+ . Arteries isolated from patients with type 1 diabetes demonstrated a significantly increased sensitivity to ET-1 compared with controls, but no alteration in the maximum contraction (Figure 6.2b, Table 6.3)). In contrast, size and sensitivity of contractile responses to NA and KPSS were similar in arteries isolated from patients with type 1 diabetes and control subjects (Figures 6.2a & c; Table 6.3).

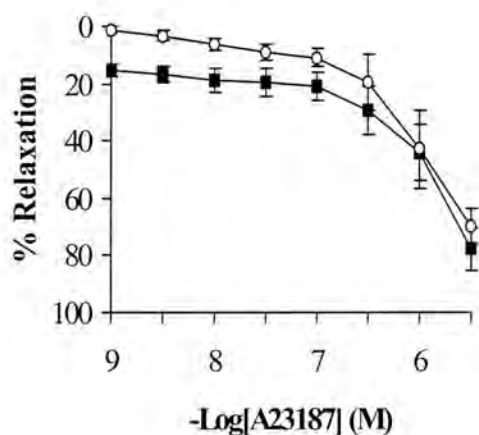
(a) Acetylcholine



(b) Bradykinin



(c) A23187



(d) SIN-1

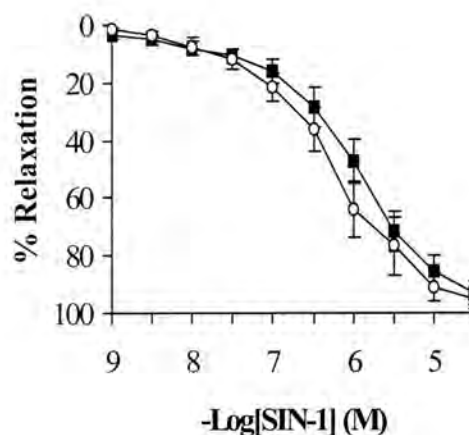


Figure 6.1. Cumulative concentration-response curves to the vasodilator agonists (a) acetylcholine (ACh; n=12), (b) bradykinin (BK; n=8), (c) A23187 (n=8-10) and (d) SIN-1 (n=11-12) in human resistance arteries isolated from patients with type 1 diabetes (○) and from non-diabetic controls (■). Results as expressed as % relaxation of vessels initially precontracted with 3μM NA and shown as mean ± s.e.mean.

Table 6.2. Maximum relaxation (% reversal of 3 μ M NA-induced tone) and sensitivity (-LogIC₅₀ values) from concentration-response curves obtained to the vasodilator agonists in arteries from patients with type 1 diabetes and controls.

	Maximum Relaxation (%)			Sensitivity (-logIC ₅₀)		
	Control	Type 1	<i>P</i>	Control	Type 1	<i>P</i>
ACh	97.2 \pm 1.0	95.8 \pm 1.8	0.50	7.30 \pm 0.09	7.23 \pm 0.12	0.63
BK	94.3 \pm 2.2 (8)	91.7 \pm 1.9 (8)	0.38	7.69 \pm 0.15 (8)	7.93 \pm 0.15 (8)	0.29
A23187	73.3 \pm 3.2 (10)	61.2 \pm 12.3 (8)	0.12	5.37 \pm 0.17 (10)	6.10 \pm 0.16 (8)	0.61
SIN-1	93.8 \pm 4.4	95.5 \pm 3.2	0.76	5.87 \pm 0.21	6.22 \pm 0.22	0.26

All values are mean \pm s.e.mean, n=8-12, unless indicated otherwise by values in parentheses. Sensitivity values for A23187 are an approximate as the response to this agonist did not reach a maximum in the concentration range used. ACh, acetylcholine, BK, bradykinin, SIN-1, 3'morpholinosydnonimine.

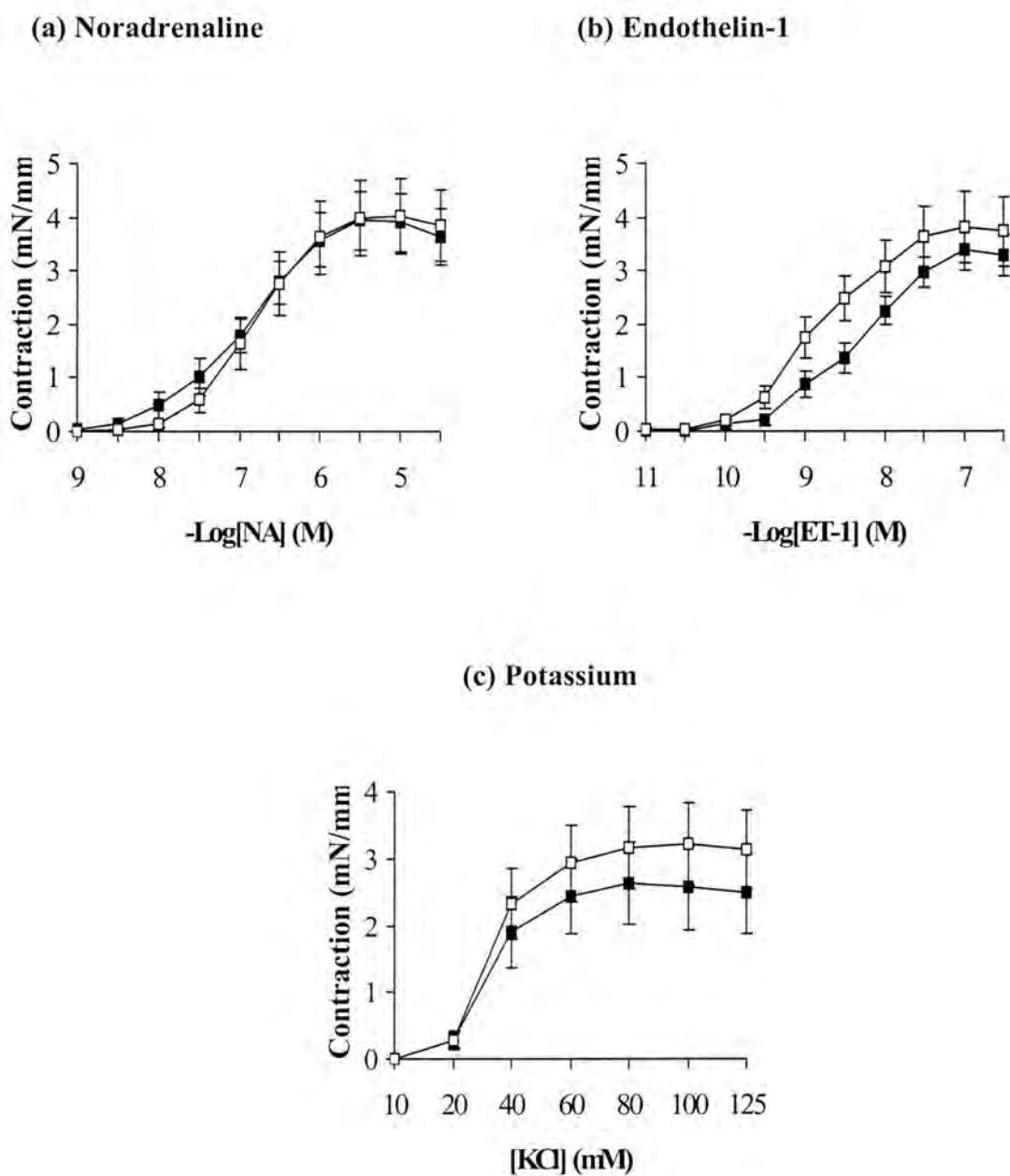


Figure 6.2. Cumulative concentration-response curves to vasoconstrictor agonists (a) endothelin-1 (ET-1), (b) noradrenaline (NA) and (c) potassium (K^+) in human resistance arteries isolated from patients with type 1 diabetes (\square) and non-diabetic controls (\blacksquare). Each point represents mean \pm s.e.mean, $n=12$.

Table 6.3. Maximum contraction (a) and sensitivity (b) values from concentration-response curves obtained to the vasoconstrictor agonists in arteries isolated from patients with type 1 diabetes and non-diabetic controls.

(a)

	Maximum Contraction (mN/mm)			Maximum Contraction (%NA-K)		
	Control	Type 1	<i>P</i>	Control	Type 1	<i>P</i>
NA	3.97±0.54	4.05±0.71	0.93	115.4±3.2	108.3±2.4	0.09
K ⁺	2.67±0.47	3.26±0.61	0.46	71.9±6.7	85.0±7.7	0.21
ET-1	3.39±0.39	3.84±0.65	0.56	103.7±5.9	104.5±6.0	0.92

All values are mean ± s.e.mean, n=12.

(b)

	Sensitivity (pD ₂)		
	Control	Type 1	<i>P</i>
NA	6.98±0.14	6.73±0.11	0.19
K ⁺	1.47±0.03	1.50±0.04	0.55
ET-1	8.35±0.13	8.87±0.12	0.008

All values are mean ± s.e.mean, n=12.

6.4.2. *Influence of Elevated Glucose Concentrations*

The details of the control subjects and patients with Type 1 diabetes who provided arteries for exposure to elevated glucose are given in Table 6.4). As with the larger group, non-diabetic controls and diabetic patients were well matched but glucose and glycosylated haemoglobin concentrations were elevated in the latter. Urinary albumin/creatinine ratios for the type 1 diabetic patients ($1.76 \pm 0.36 \text{ mg/mmol}$) were within the normal range ($0.0\text{-}3.5 \text{ mg/mmol}$), indicating normal renal function. The gluteal resistance arteries isolated from patients (mean internal diameter control incubation, $284 \pm 28 \mu\text{m}$; glucose incubation, $268 \pm 22 \mu\text{m}$) were larger than those obtained from controls (control incubation, $187 \pm 17 \mu\text{m}$, $P=0.016$; glucose incubation, $190 \pm 29 \mu\text{m}$, $P=0.05$).

6.4.2.1. Responses to Vasodilators

Incubation with 20mM glucose for 1 hour did not alter the ability of resistance arteries from control subjects to respond to either the endothelium-dependent (ACh, BK) or –independent (SIN-1) vasodilators (Figure 6.3; Table 6.5a). Endothelium-dependent relaxation was also unaffected in arteries from patients with type 1 diabetes following exposure to elevated glucose, whereas the sensitivity of these vessels to SIN-1 was enhanced (Figure 6.4; Table 6.5b).

6.4.2.2. Responses to Vasoconstrictors

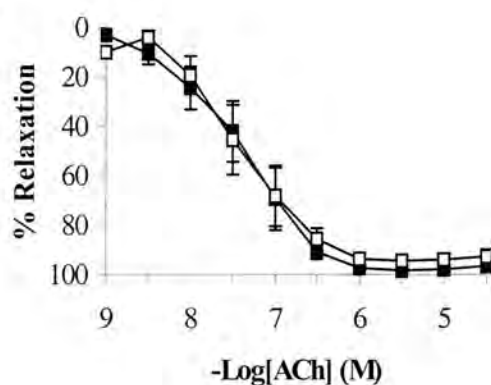
Incubation with 20mM glucose had no effect on the contractile responses of arteries from non-diabetic controls (Figure 6.5; Table 6.6) and patients with type 1 diabetes (Figure 6.6; Table 6.7).

Table 6.4. Characteristics of non-diabetic control subjects (n=6) and patients with type 1 diabetes (n=8) who provided vessels for incubation with control (5.5mM) and elevated (20mM) glucose.

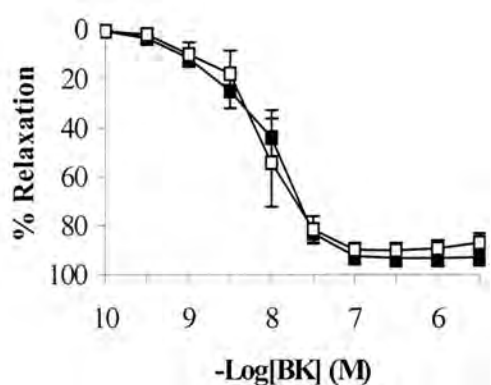
	CONTROL	Type 1	P
Age (yrs)	28.5±2.6	30.5±1.5	0.49
Duration type 1 Diabetes (yrs)	-	9.8±2.3	-
Blood Pressure (mmHg) Systolic	120.0±4.09	120.2±4.7	0.97
Diastolic	74.8±2.8	74.9±0.99	0.99
Glucose (mmol/l)	4.82±0.10	14.31±1.71	<0.0005
Glycosylated Hb (%)	5.02±0.20	9.45±0.46	<0.000005
Serum Cholesterol (mmol/l)	4.47±0.70	5.24±0.31	0.29
Serum Triacylglycerol (mmol/l)	1.12±0.31	1.89±0.54	0.29
Serum Creatinine (μmol/l)	74.5±2.7	66.9±3.4	0.12
Serum Albumin (g/l)	43.67±1.43	36.71±5.81	0.30
Urea (mmol/l)	4.58±0.26	4.63±0.37	0.93

Values are shown as mean ± s.e.mean.

(a) Acetylcholine



(b) Bradykinin



(c) SIN-1

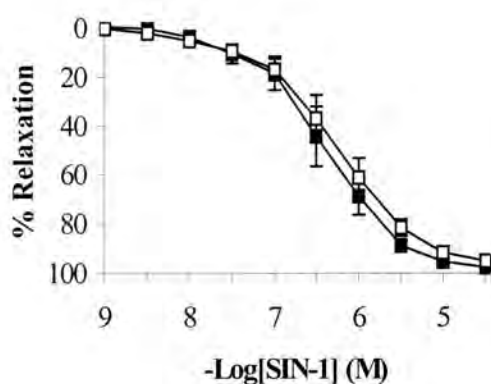
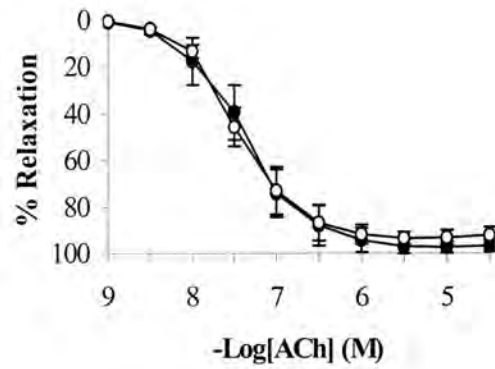
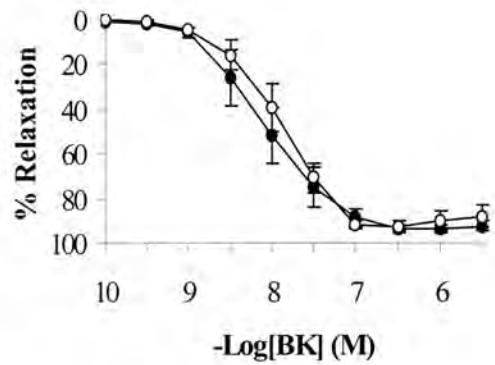


Figure 6.3. Cumulative concentration-response curves to the vasodilator agonists (a) acetylcholine (ACh), (b) bradykinin (BK) and (c) SIN-1, in resistance arteries isolated from healthy volunteers and incubated in the presence of 5.5mM (Control, ■ n=6) or 20mM (□, n=6) glucose. Results as expressed as % relaxation of vessels initially precontracted with 3μM NA and shown as mean ± s.e.mean.

(a) Acetylcholine



(b) Bradykinin



(c) SIN-1

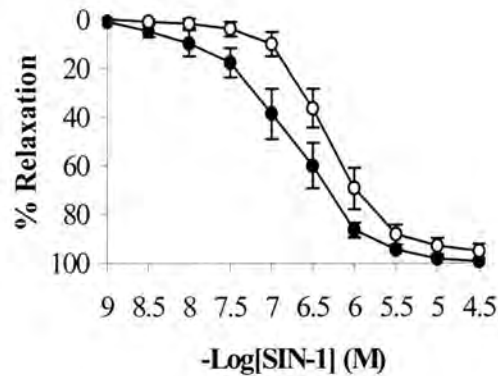


Figure 6.4. Cumulative concentration-response curves to the vasodilator agonists (a) acetylcholine (ACh), (b) bradykinin (BK) and (c) SIN-1 in resistance arteries isolated from patients with type 1 diabetes incubated in the presence of 5.5mM (●; control) and 20mM (○; elevated) glucose. Results are expressed as % relaxation of vessels initially precontracted with 3μM NA and shown as mean ± s.e.mean, n=8.

Table 6.5. Maximum relaxation (% reversal of 3 μ M NA-induced tone) and sensitivity (-LogIC₅₀ values) from concentration-response curves obtained to the vasodilator agonists in arteries from (a) non-diabetic control subjects (n=6) and (b) patients with type 1 diabetes (n=8) after incubation with either normal (5mM) or elevated (20mM) glucose.

(a) Non-Diabetic Controls

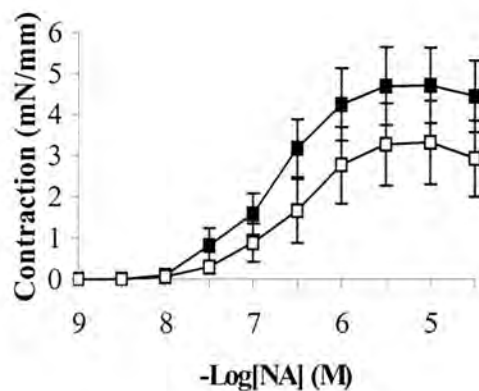
	Maximum Relaxation (%)			Sensitivity (-logIC ₅₀)		
	Normal Glucose	High Glucose	<i>P</i>	Normal Glucose	High Glucose	<i>P</i>
ACh	98.6 \pm 1.0	94.6 \pm 1.9	0.09	7.45 \pm 0.22	7.43 \pm 0.20	0.94
BK	93.2 \pm 3.3	92.9 \pm 1.5	0.94	8.06 \pm 0.12	8.10 \pm 0.16	0.86
SIN-1	97.7 \pm 1.0	95.0 \pm 2.1	0.26	6.46 \pm 0.18	6.28 \pm 0.14	0.46

(b) Type 1 Diabetics

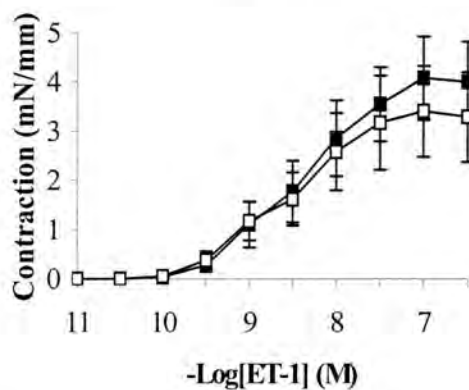
	Maximum Relaxation (%)			Sensitivity (-logIC ₅₀)		
	Normal Glucose	High Glucose	<i>P</i>	Normal Glucose	High Glucose	<i>P</i>
ACh	98.6 \pm 2.1	95.2 \pm 1.8	0.25	7.35 \pm 0.20	7.36 \pm 0.16	0.94
BK	94.0 \pm 1.7	93.4 \pm 2.0	0.36	8.04 \pm 0.19	7.89 \pm 0.13	0.52
SIN-1	99.2 \pm 1.0	95.1 \pm 3.1	0.24	6.81 \pm 0.17	6.32 \pm 0.11	0.03

All values are mean \pm s.e.mean. ACh, acetylcholine, BK, bradykinin, SIN-1, 3'-morpholinonydnonimine.

(a) Noradrenaline



(b) Endothelin-1



(c) Potassium

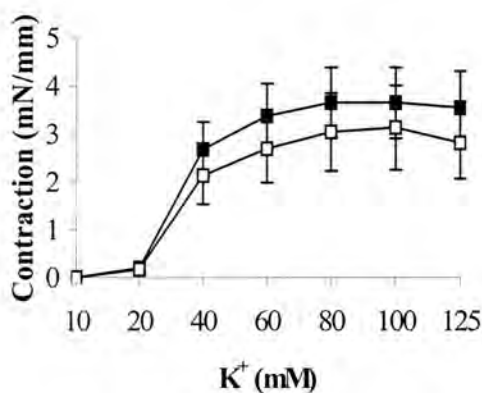


Figure 6.5. Cumulative concentration-response curves to vasoconstrictor agonists (a) noradrenaline (b) endothelin-1 and (c) high potassium, in resistance arteries isolated from healthy volunteers and incubated in the presence of 5.5 mM (Control, ■ n=6) or 20 mM (□, n=6) glucose for 1 hour. Results are expressed as force per unit length of tissues (mN/mm) and shown as mean \pm s.e.mean.

Table 6.6. Maximum contraction (a) and sensitivity (b) values from concentration-response curves obtained to the vasoconstrictor agonists in arteries isolated from non-diabetic controls incubated with either normal (5mM) or high (20mM) glucose for 1 hour.

(a)

	Maximum Contraction (mN/mm)			Maximum Contraction (%NA-K)		
	Normal	High	<i>P</i>	Normal	High	<i>P</i>
NA	4.75±0.93	3.34±1.01	0.33	108.3±5.5	112.6±19.1	0.84
K ⁺	3.72±0.73	3.16±0.88	0.63	84.5±6.0	116.6±30.0	0.31
ET-1	4.09±0.84	3.41±0.92	0.60	94.1±6.8	135.3±41.3	0.34

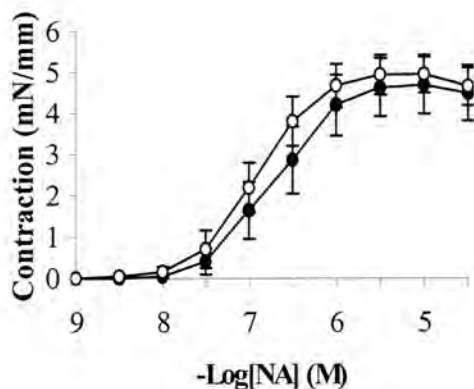
All values are mean ± s.e.mean, n=6.

(b)

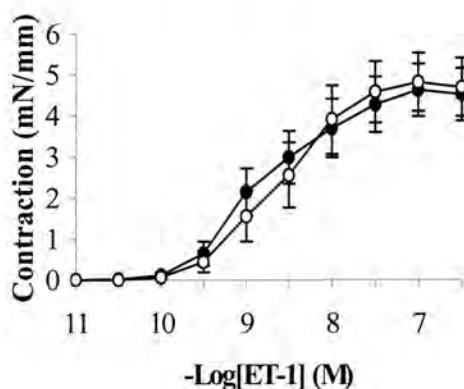
	Sensitivity (pD ₂)		
	Normal Glucose	High Glucose	<i>P</i>
NA	6.81±0.18	6.55±0.16	0.32
K ⁺	1.47±0.04	1.48±0.02	0.69
ET-1	8.31±0.20	8.49±0.19	0.52

All values are mean ± s.e.mean, n=6.

(a) Noradrenaline



(b) Endothelin-1



(c) Potassium

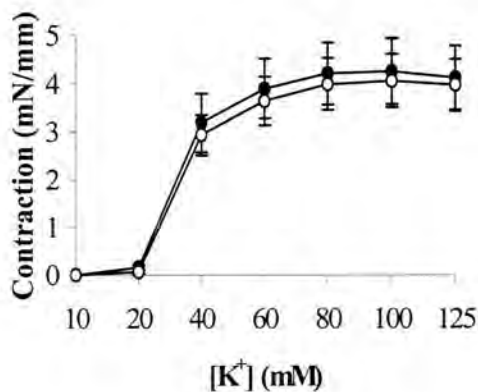


Figure 6.6. Cumulative concentration-response curves to vasoconstrictor agonists (a) noradrenaline (b) endothelin-1 and (c) high potassium, in resistance arteries isolated from patients with type 1 diabetes and incubated in the presence of 5.5mM (Control, \bigcirc $n=8$) or 20mM (\bullet , $n=8$) glucose. Results are expressed as force per unit length of tissues (mN/mm) and shown as mean \pm s.e.mean.

Table 6.7. Maximum contraction (a) and sensitivity (b) values from concentration-response curves obtained to the vasoconstrictor agonists in arteries isolated from patients with type 1 diabetes incubated with either normal (5mM) or high (20mM) glucose for 1 hour.

(a)

	Maximum Contraction (mN/mm)			Maximum Contraction (%NA-K)		
	Normal	High	<i>P</i>	Normal	High	<i>P</i>
NA	4.80±0.69	5.00±0.47	0.81	104.9±2.3	113.0±6.0	0.23
K ⁺	4.33±0.66	4.10±0.57	0.79	95.4±5.7	87.8±3.6	0.28
ET-1	4.70±0.63	4.87±0.70	0.86	105.9±6.5	106.4±4.25	0.95

All values are mean ± s.e.mean, n=8.

(b)

	Sensitivity (pD ₂)		
	Normal Glucose	High Glucose	<i>P</i>
NA	6.67±0.15	6.87±0.12	0.32
K ⁺	1.48±0.02	1.49±0.02	0.87
ET-1	8.77±0.21	8.57±0.21	0.51

All values are mean ± s.e.mean, n=8.

6.4.3. *Type 2 Diabetes*

Type 2 diabetic patients were well matched with their controls in all respects, with the single exception that HbA1c levels were slightly, but significantly, elevated in the diabetic group (Table 6.8). The resistance arteries isolated from gluteal biopsies taken from patients (mean internal diameter, $161 \pm 10 \mu\text{m}$) were similar in size ($P=0.13$) to those isolated from controls ($190 \pm 14 \mu\text{m}$).

6.4.3.1. Responses to Vasodilators

In accordance with the study of type 1 diabetes, there was no evidence of endothelial cell dysfunction in resistance arteries from patients with type 2 diabetes. The responses produced by ACh, BK, and A23187 were similar in arteries from patients with type 2 diabetes and controls (Figure 6.7; Table 6.9). There was a tendency towards a reduced response (maximum relaxation and sensitivity) to SIN-1 in arteries from diabetic patients but this did not achieve significance (Figure 6.7; Table 6.9).

6.4.3.2. Responses to Vasoconstrictors

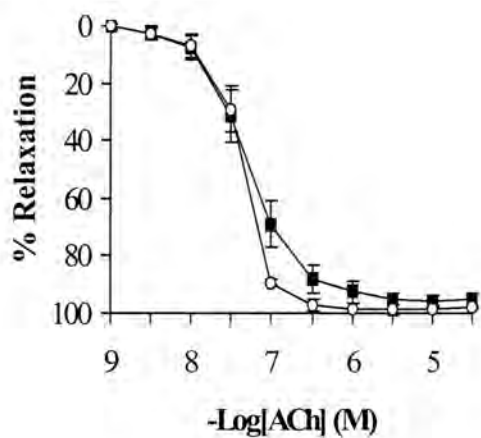
The magnitude of contraction (E_{max}) produced in arteries from patients with type 2 diabetes tended to be smaller than those produced in control arteries for all three vasoconstrictors (Figure 6.8). This difference, however, only achieved significance in the case of potassium. The presence of type 2 diabetes had no effect on sensitivity to vasoconstrictors, with no evidence of an increase in sensitivity to ET-1 similar to that seen in patients with type 1 diabetes.

Table 6.8. Baseline characteristics of 8 male patients with type 2 diabetes and 10 age and sex-matched non-diabetic control subjects.

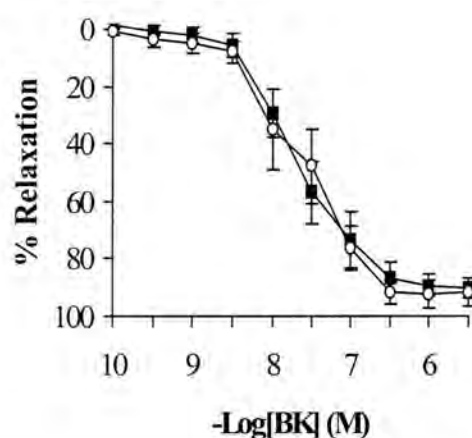
	CONTROL	Type 2	<i>P</i>
Age (yrs)	62.1±2.3	58.9±2.4	0.36
Sex (M:F)	10:0	8:0	
Smoker (Y:N)	2:8	4:4	
Blood Pressure (mmHg)	135.30±4.18	138.75±4.09	0.93
	78.70±2.57	77.50±2.47	0.50
BMI	28.03±0.61	26.78±1.09	0.31
Glucose (mmol/l)	5.41±0.25	6.31±0.76	0.29
HbA1c (%)	6.11±0.15	6.63±0.19	0.046
Triglycerides (mmol/l)	2.11±0.42	2.84±0.41	0.23
Cholesterol (mmol/l)	5.19±0.14	4.91±0.24	0.31

Values are shown as mean ± s.e.mean. BMI, body mass index;

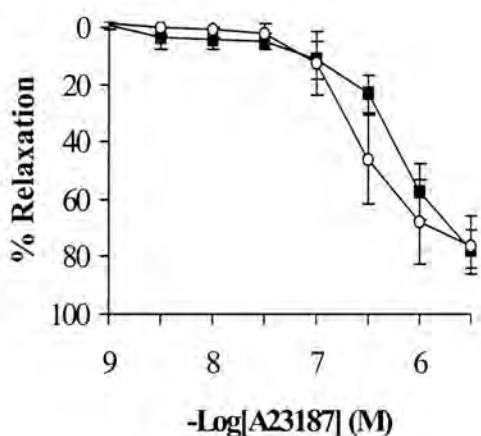
(a) Acetylcholine



(b) Bradykinin



(c) A23187



(d) SIN-1

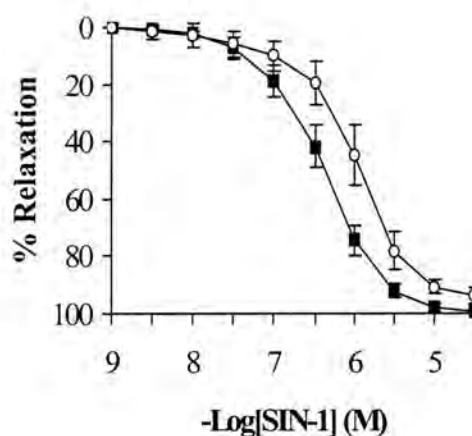


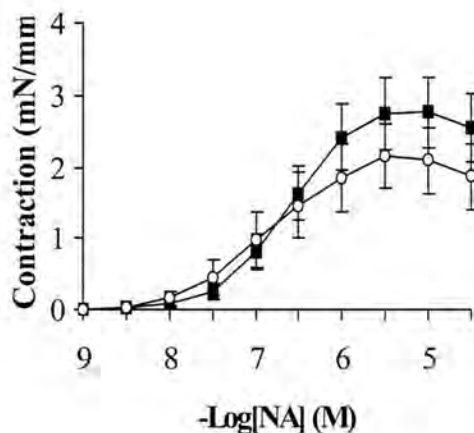
Figure 6.7. Cumulative concentration-response curves to the vasodilator agonists (a) acetylcholine, (b) bradykinin, (c) A23187 and (d) SIN-1 in human resistance arteries isolated from patients with type 2 diabetes (○, n=8) and non-diabetic controls (■, n=10). Results as expressed as % relaxation of vessels initially precontracted with 3μM NA and shown as mean ± s.e.mean.

Table 6.9. Maximum relaxation (% reversal of 3 μ M NA-induced tone) and sensitivity (-LogIC₅₀ values) from concentration-response curves obtained to the vasodilator agonists in the arteries from patients with type 2 diabetes and controls.

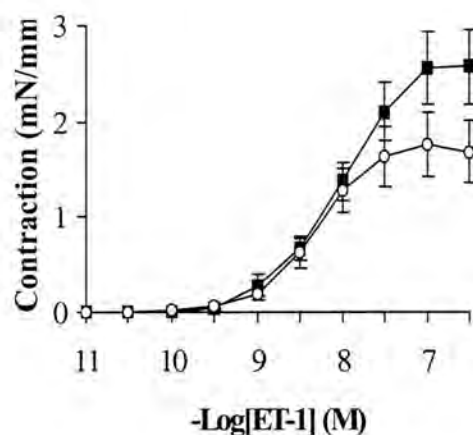
	Maximum Relaxation (%)			Sensitivity (-logIC ₅₀)		
	Control	Type 2	<i>P</i>	Control	Type 2	<i>P</i>
ACh	95.9 \pm 2.0	99.4 \pm 1.4	0.19	7.28 \pm 0.10	7.37 \pm 0.06	0.48
BK	90.7 \pm 3.2	93.0 \pm 4.1	0.67	7.64 \pm 0.16	7.54 \pm 0.16	0.67
A23187	78.4 \pm 6.7	79.6 \pm 10.5	0.92	6.22 \pm 0.07	6.52 \pm 0.16	0.10
SIN-1	99.5 \pm 1.1	94.7 \pm 2.3	0.06	6.43 \pm 0.13	6.05 \pm 0.14	0.07

All values are mean \pm s.e.mean, n=8-12, unless indicated otherwise by values in parentheses. Sensitivity values for A23187 are an approximate as the response to this agonist did not reach a maximum in the concentration range used. ACh, acetylcholine, BK, bradykinin, SIN-1, 3'morpholinosydnonimine.

(a) Noradrenaline



(b) Endothelin-1



(c) Potassium

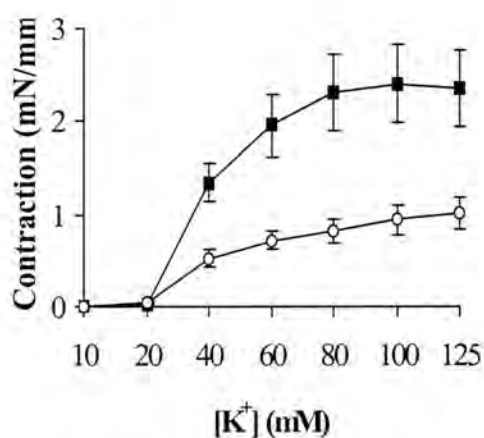


Figure 6.8. Cumulative concentration-response curves to vasoconstrictor agonists (a) noradrenaline (b) endothelin-1 and (c) high potassium, in resistance arteries isolated from patients with type 2 diabetes (○, n=8) and non-diabetic controls (■, n=10). Results are expressed as force per unit length of tissues (mN/mm) and shown as mean \pm s.e.mean.

Table 6.10. Maximum contraction (a) and sensitivity (b) to vasoconstrictor agonists in isolated resistance arteries from patients with type 2 diabetes (n=8) and non-diabetic control subjects (n=10).

(a)

Maximum Contraction (mN/mm)			
	Control	Type 2	<i>P</i>
NA	2.76±0.50	2.20±0.45	0.43
ET-1	2.61±0.39	1.79±0.33	0.14
K ⁺	2.43±0.42	1.04±0.17	0.01

(b)

Sensitivity (pD ₂)			
	Control	Type 2	<i>P</i>
NA	6.66±0.09	6.71±0.18	0.80
ET-1	8.18±0.16	8.37±0.09	0.36
K ⁺	1.56±0.04	1.59±0.06	0.69

Values are expressed as mean ± s.e.mean. NA, noradrenaline, ET-1, endothelin-1.

6.5. Discussion

This investigation has made two striking observations in resistance arteries isolated from patients with diabetes: (1) there is a selective alteration of contractile function, dependent on the type of diabetes and (2) endothelium-dependent (and endothelium-independent) relaxation is unaffected by either type 1 or type 2 diabetes.

6.5.1. Type 2 diabetes

Endothelium-dependent and independent vasodilatation was assessed in resistance arteries isolated from patients with type 2 diabetes and control subjects. In this selected group of well-controlled type 2 patients, responses to receptor-dependent (ACh, BK) and receptor-independent (A23187), endothelium-dependent dilators were unaltered in the present study. These results contrast with previous findings demonstrating reduced endothelium-dependent and independent responses in type 2 diabetic patients (McVeigh *et al.*, 1992; Morris *et al.*, 1995; Watts *et al.*, 1996). This discrepancy may be due to the inclusion of patients with more severe hyperglycaemia and dyslipidaemia in previous studies, whereas this study included only well-controlled type 2 patients. On the other hand, these findings are well in agreement with previous studies demonstrating unaltered endothelium-dependent relaxation in patients with uncomplicated type 2 diabetes (Avogaro *et al.*, 1997; Cockcroft *et al.*, 1998). These results indicate, therefore, that overall, altered blood insulin and glucose levels during the development and progression of type 2 diabetes do not result in impaired function of microvascular endothelium.

The tendency towards a reduced response to the endothelium-independent vasodilator, SIN-1, did not achieve significance; a factor which may be due to type 2 error, despite good group numbers (n=8 and 10), as the power to detect a difference was 40-50%. Reduced responses to endothelium-independent dilators have been reported in the forearm of patients with type 2 diabetes (McVeigh *et al.*, 1992; Morris *et al.*, 1995). These results, however, contrast with the majority of studies in which endothelium-independent relaxation is unaltered (Cockcroft *et al.*, 1998; Avogaro *et al.*, 1997; Cipolla *et al.*, 1996) or even enhanced (Lawrence *et al.*, 1994).

Only the contractile responses to the receptor-independent vasoconstrictor K^+ were diminished significantly in resistance arteries from patients with Type 2 diabetes. This was not due to a general inability of the smooth muscle cells to contract as responses to the receptor-dependent vasoconstrictors, NA and ET-1, were not altered. This observation is in agreement with previous studies in which contractile responses evoked by K^+ depolarization have been found to be reduced in diabetic vessels (Fulton *et al.*, 1991; Hattori *et al.*, 1996). The diminished contractile response of diabetic rat aorta to high K^+ appears to be associated with reduced Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Therefore, it is possible that activation of Ca^{2+} channels by membrane depolarisation may be specifically impaired in diabetes.

6.5.2. *Type 1 diabetes*

A selective enhancement of the response to the endothelium-derived contracting factor, ET-1, was detected in resistance arteries from patients with Type 1 diabetes. Whilst altered receptor activity in the endothelium may contribute to this

abnormality, it was strikingly evident that endothelium-dependent relaxation was unaltered in these arteries. These results indicate that endothelial cell function is largely maintained in sub-cutaneous resistance arteries from patients with Type 1 diabetes and no detectable microvascular complications.

Damage to the endothelium often results in increased release of ET-1. Consequently, the elevated plasma (Takahashi *et al.*, 1990) and tissue (Properzi *et al.*, 1995) concentrations of this peptide in patients suggest that damage to the endothelium is a feature of Type 1 diabetes. Similarly, urinary concentrations of ET-1 are elevated (Morabito *et al.*, 1995), and its release from resistance arteries is enhanced (Takeda *et al.*, 1991), in diabetic rats whilst increased release of ET-1 possibly contributes to the down-regulation of glomerular ET receptors (Awazu *et al.*, 1991). The precise role of ET-1 in the development of vascular complications, however, remains unclear. Functional data obtained using arteries from diabetic rats are contradictory, and there is evidence that the duration of diabetes has a significant impact on both ET-1-mediated contraction and endothelial cell function (Hopfner & Gopalakrishnan, 1999).

The selective enhancement of ET-1-mediated contraction in arteries from patients with Type 1 diabetes suggests altered function of the ET receptors in the endothelium and /or smooth muscle cells. This contrasts with studies using diabetic animals in which increased ET-1-mediated contraction was just one component of a non-specific enhancement of contractile function, possibly mediated by altered second messenger activity (White & Carrier, 1990; Hopfner *et al.*, 1999). Unaltered

responses to NA and KPSS in the present study indicated that vascular remodelling, or a general abnormality in second messenger systems, were not responsible for the increased response to ET-1. Endothelium-derived relaxing factors have a modulatory effect on ET-1-mediated contraction of human sub-cutaneous resistance arteries (Buckley *et al.*, 2002). The current investigation suggests that this function of the endothelium has been lost in arteries from patients with Type 1 diabetes. As the ability of the endothelium to mediate relaxation was not impaired, this may suggest down-regulation of endothelial ET_B receptors. This is strikingly similar to results obtained using resistance arteries from patients with hypertension (Angus *et al.*, 1992) and normal pressure glaucoma (Buckley *et al.*, 2002). Alternatively, the enhanced ET-1-mediated contraction may be a consequence of up-regulation of ET_A and/ or ET_B receptors on the vascular smooth muscle cells. Further studies would be required to identify the exact mechanism(s) responsible for the enhanced response to ET-1.

The demonstration of unaltered responses to NA is consistent with data obtained in the isolated fore-arm (Calver *et al.*, 1992; Johnstone *et al.*, 1993) and in resistance arteries from patients with Type 1 diabetes (Malik *et al.*, 1999). Increased AII-mediated contraction in the latter supports the contention that contractile abnormalities detected in these arteries are the result of specific alterations in receptor function. Furthermore, although a non-specific reduction in contractile function (using NA, AII and KPSS) has also been reported in resistance arteries this may have been due to a slightly (but not significantly) lower media-lumen ratio in arteries from the diabetic group (McNally *et al.*, 1994).

Endothelial cell dysfunction in animal models of diabetes has been indicated by impaired endothelium-dependent relaxation in conduit and resistance arteries (Chan *et al.*, 2000). Similar abnormalities have also been reported in the fore-arm (Johnstone *et al.*, 1993) and brachial arteries (Zenere *et al.*, 1995) of patients with Type 1 diabetes. These results are challenged, however, by similar investigations which have found responses to endothelium-dependent relaxants to be unchanged (Smits *et al.*, 1993; Lambert *et al.*, 1996) or even enhanced (Makimattila *et al.*, 1997). The unaltered responses to receptor-dependent (ACh, BK) and receptor-independent (A23187), endothelium-dependent dilators in the present study demonstrated that endothelium-dependent relaxation was not impaired. Furthermore, the maintained response to SIN-1 indicated that the ability of the vascular smooth muscle to relax was similarly unaffected. These results are consistent with studies in which endothelium-dependent relaxation in the human fore-arm is only impaired in diabetes if patients also exhibit microalbuminuria (Calver *et al.*, 1992; Smits *et al.*, 1993; Elliot *et al.*, 1993). Furthermore, relaxation in response to NO donors (usually sodium nitroprusside) has been shown repeatedly (although not exclusively (Malik *et al.*, 1999; Calver *et al.*, 1992)) to be unaltered in patients with this condition (Johnstone *et al.*, 1993; Smits *et al.*, 1993; Lambert *et al.*, 1996; Elliot *et al.*, 1993). Therefore, it is probable that impaired endothelium-derived nitric oxide activity in the microvasculature is restricted to patients with evidence of nephropathy. This would correspond with the demonstration that microvascular permeability is only increased in patients with long-standing Type 1 diabetes if they also have severe microvascular complications (Jaap *et al.*, 1996).

These results challenge a previous demonstration that ACh-mediated relaxation is selectively impaired in resistance arteries isolated from patients with Type 1 diabetes (McNally *et al.*, 1994). The difference between these studies is unlikely to be due to differences in disease duration in diabetic groups. The duration of diabetes in the group with impaired ACh-mediated relaxation (18yrs) was shorter than in patients who demonstrated normal responses to this agonist (24 yrs). In contrast to the present study, the patient group used by McNally *et al.* (1994) had evidence of microvascular complications (retinopathy), possibly indicating that endothelium-dependent relaxation is only altered in patients with both diabetes and microvascular disease. Methodological variations are, however, a more likely reason for these discrepant results. In particular, the functional difference detected between large and small resistance arteries obtained from sub-cutaneous biopsies (Chapter 5) may be important. The inadvertent inclusion of a small number of these arteries in the diabetic group could explain both the specific reduction in ACh-mediated relaxation and the variable size and sensitivity of this response reported in the study by McNally *et al.* (1994).

6.5.2.1. Influence of Elevated Glucose Concentrations

Incubation of human sub-cutaneous resistance arteries with elevated glucose demonstrated that arteries from non-diabetic controls and patients with type 1 diabetes responded differently to glucose exposure.

Incubation with elevated glucose had no effect on either contractile or relaxation of arteries from non-diabetic controls. The inability of glucose exposure to impair

endothelium-dependent relaxation contrasts with studies using rat mesenteric artery (Taylor & Poston, 1994) and rabbit aorta (Tefamariam *et al.*, 1990). As discussed previously, this lack of effect may be a result of the short incubation time (1hr), the relatively low concentration of glucose (20mM rather than the 40mM concentrations used in some studies; Heygate *et al.*, 1993), or the contribution of EDHF to relaxation of resistance arteries (see Chapter 5). It should also be noted that 20mM glucose failed to cause a significant change in reactivity of rat mesenteric arteries; therefore, it would be interesting to assess the influence of 15mM or 44mM glucose but there were not enough vessels available. The presence of type 1 diabetes had no effect on the influence of elevated glucose on contraction or endothelium-dependent relaxation of resistance arteries. This indicated, therefore, that the sensitivity of contractile function and endothelium-dependent relaxation to glucose exposure was not altered in resistance arteries from patients with diabetes.

The demonstration that resistance arteries from patients with type 1 diabetes were more sensitive than controls to ET-1 was striking given the reduction in sensitivity to this agonist following incubation of rat mesenteric arteries with elevated glucose (Chapter 4). One possibility is that the inhibitory effect of hyperglycaemia *in vivo* causes a compensatory enhancement of the response to ET-1 which becomes apparent in isolated vessels *in vitro*. It was hypothesised, therefore, that the combination of the inhibitory effect of glucose with the enhanced contractility in diabetic arteries would return the sensitivity of resistance arteries from patients with type 1 diabetes towards control values. In fact, however, glucose did not alter ET-1

mediated contraction of human resistance arteries and had no ameliorating effect on the enhanced sensitivity to this agonist in arteries from the diabetic group.

The only difference in the response of resistance arteries from diabetic patients and controls to elevated glucose was observed with the endothelium-independent relaxation induced by SIN-1. The enhanced sensitivity to SIN-1 in the presence of glucose suggests an increased sensitivity of the vascular smooth muscle. The mechanisms through which exposure to glucose would enhance peroxynitrite-mediated relaxation are not clear and would require further investigation.

6.5.3. *Conclusions*

The work reported in this chapter has shown that the presence of diabetes is associated with altered contractile function in subcutaneous resistance arteries. The nature of this alteration is vasoconstrictor-selective and depends upon whether the patient has type 1 or type 2 diabetes. The enhanced response to ET-1 in the type 1 study may be caused by an impairment of the ability of the endothelium to modulate the response to this agonist. It was striking that a short-term elevation of glucose concentration had no effect on ET-1-mediated contraction (in either diabetic or control subjects) suggesting that the altered response to this agonist is not the result of acute hyperglycaemia. In contrast, the reduction in potassium-mediated contraction in the type 2 diabetic group suggests an alteration in contractile regulation within the smooth muscle cells. Arguably the most striking result is the demonstration that endothelium-dependent relaxation is not impaired in either form of diabetes. This contrasts with large vessel studies which generally report impaired

NO activity. It is possible, therefore, that the EDHF-mediated component of relaxation in small arteries protects against the endothelial cell dysfunction caused by diabetes in conduit arteries. The possibility that the balance of endothelium-derived relaxing factors has altered in patients with type 1 diabetes is supported by the demonstration that SIN-1-mediated relaxation was sensitive to elevated glucose in resistance arteries from these patients. It remains possible that endothelial cell function is altered in resistance arteries in other vascular territories and also in subcutaneous arteries from patients with uncontrolled diabetes and attendant microvascular complications.

GENERAL DISCUSSION

Type 1 and Type 2 diabetes mellitus are associated with abnormal vascular function, but few studies have documented their effects on human resistance arteries. The primary aim of the research described in this thesis was to determine whether endothelial cell and vascular smooth muscle cell function were altered in resistance arteries isolated from patients with these conditions. The results from this investigation clearly demonstrated normal endothelium-dependent and endothelium-independent relaxation in resistance arteries from both Type 1 and Type 2 diabetic subjects. In contrast, however, there was a selective alteration of contractile function, which was dependent on the type of diabetes. Arteries isolated from Type 1 diabetic patients demonstrated a selective enhancement of ET-1-induced contraction, whilst a selective attenuation of contractile responses to K^+ was demonstrated in arteries isolated from patients with Type 2 diabetes. The variable results between the two groups could be attributed not only to the different type of diabetes but also to variations in a number of factors, and may be partially explained by differences in the duration and degree of the diabetic state, degree of acute hyperglycaemia, age and lipid profile.

The enhanced ET-1-mediated contraction in arteries from patients with type 1 diabetes contrasted with the ability of elevated glucose to inhibit such contractions in rat mesenteric arteries. Short-term exposure of arteries from type 1 diabetic patients to 20mM glucose did not, however, normalise the response to ET-1 in these vessels. This suggests that the enhanced contractility in these patients is not simply an adaptive response to an inhibitory effect of short-term hyperglycaemia.

The demonstration of unaltered endothelium-dependent relaxation suggests that endothelial cell function is largely intact in arteries from patients with established, but well controlled, diabetes. This contrasts directly with the general consensus that the ability of the endothelium to mediate vascular relaxation is impaired in large conduit arteries from patients and animals with diabetes mellitus. Receptor populations differ in arteries of different anatomical origin and, perhaps more importantly, the factors that contribute to endothelium-dependent relaxation differ in large compared with small vessels. This investigation confirmed functional differences between large and small resistance arteries obtained from biopsies of human, subcutaneous, gluteal fat, with large resistance arteries unable to relax in response to the endothelium-dependent vasorelaxant, ACh. These results indicate a heterogeneity in endothelial cell function between large and small resistance arteries from human gluteal fat, which may be the result of variations in receptor population. This emphasises the need to ensure careful selection of arteries from this source, which can be achieved using physical and functional criteria identified, when performing functional investigations. Relaxation in response to ACh results from the release of relaxing factors following stimulation of muscarinic receptors on the endothelium. In large conduit arteries, endothelium-dependent relaxation is mediated almost exclusively by NO, whereas in smaller arteries, both NO and EDHF may contribute. This investigation demonstrated that ACh-induced endothelium-dependent relaxation of human subcutaneous resistance arteries is mediated by both NO-dependent and NO-independent pathways. The NO/PG-independent response to ACh, which accounted for the majority of the relaxation to ACh, had characteristics consistent with EDHF-mediated relaxation. It is probable that the impaired

endothelium-dependent relaxation generally observed in large conduit arteries in diabetes results from reduced NO activity/availability. This attenuation was not observed in resistance vessels in the present study and is probably due to the fact that, unlike large conduit arteries, NO has a limited role as a mediator of endothelium-dependent relaxation in these vessels.

The exact nature of vascular alterations in diabetes is not completely understood. However, it has been demonstrated that strict glycaemic control is associated with a reduced risk of microvascular complications, suggesting that hyperglycaemia may be an initiating factor in the development of such complications. A number of studies have demonstrated that exposure of large conduit to high glucose concentrations reduces ACh-induced endothelium-dependent relaxation. In the present study, however, endothelium-dependent and -independent relaxation was not altered following exposure of rat mesenteric, or human subcutaneous, resistance arteries to acute elevations of glucose. This may be due to the different vessel types studied and the fact that the pathways implicated in hyperglycaemia-induced endothelial cell dysfunction, including the polyol pathway, free radicals, protein kinase C, all reduce NO-mediated relaxation. The observation that NO plays a limited role in the relaxation of resistance arteries may, therefore, account for the lack of effect of hyperglycaemia on endothelial cell function in the present study. The presence of type 1 diabetes did not alter the sensitivity of endothelium-dependent relaxation to glucose exposure in human resistance arteries, but did affect endothelium-independent relaxation. This suggested a change in signalling pathways within the

vascular smooth muscle and may indicate that the balance of endothelium-derived relaxing factors was altered in this condition.

The use of resistance arteries provides a valuable tool to investigate *in vitro* the physiological and pharmacological control of the vasculature and the mechanism responsible for altered vascular function in disease states. The requirement that blood vessels must be freshly isolated restricts the number of investigations which can be performed on vessel segments from a single animal and is even more problematic when using human vessels, as the availability of such vessels can be both irregular and unpredictable. Therefore, it would be advantageous if vessels could be stored prior to experimentation without adversely affecting vascular function. Using a readily available source of tissue, this study has clearly demonstrated that rat mesenteric resistance arteries maintain stable responsiveness to vasoconstrictors and the endothelium-dependent and endothelium-independent vasodilators, ACh and SIN-1, respectively, when stored for up to four days in a simple PSS medium at 4°C. This straightforward and reliable method should allow more efficient use of animal tissues in the future and maximise the number of experiments which can be performed using vessels from a human source.

In conclusion, these studies have demonstrated that, in contrast to conduit arteries, endothelium-dependent relaxation is not impaired in resistance arteries isolated from patients with either complication-free, type 1 or well-controlled, type 2 diabetes mellitus. Investigation of the mechanisms of endothelium-dependent relaxation in normal vessels of this size suggested that the contribution made by the endothelium-

derived hyperpolarizing factor protects against diabetes-induced impairment in NO activity. Furthermore, although distinct changes in contractile function were detected in type 1 and type 2 diabetic groups, these changes did not mimic the contractile changes induced by exposure to elevated glucose concentrations *in vitro*. This demonstrates that the functional changes in resistance vessels from diabetic patients are not simply the result in acute elevation of blood glucose concentrations.

REFERENCES

- AALKJAER, C., HEAGERTY, A.M., PETERSEN, K.K., SWALES, J.D. & MULVANY, M.J. (1987). Evidence for increased media thickness, increased neuronal amine uptake, and depressed excitation-contraction coupling in isolated resistance vessels from essential hypertensives. *Circ. Res.*, **61**, 181-186.
- AALKJAER, C., JOHANSEN, P., PEDERSEN, E.B., RASMUSSEN, A. & MULVANY, M.J. (1984). Morphology and angiotensin II responses of isolated resistance vessels from patients with pre-eclampsia. *Scand. J. Lab. Clin. Invest.*, **44**, 57-60.
- AALKJAER, C., PEDERSEN, E.B., DANIELSEN, H., FJELDBORG, O., JESPERSEN, B., KJAER, T., SØRENSEN, S.S. & MULVANY, M.J. (1986). Morphological and functional characteristics of isolated resistance vessels in advanced uraemia. *Clin. Sci.*, **71**, 657-663.
- ABEBE, W., HARRIS, K.H. & MACLEOD, K.M. (1990). Enhanced contractile responses of arteries from diabetic rats to α 1-adrenoceptor stimulation in the absence and presence of extracellular calcium. *J. Cardiovasc. Pharmacol.*, **16**, 239-248.
- ABIRU, T., KAMATA, K., MIYATA, N. & KASUYA, Y. (1990). Differences in vascular responses to vasoactive agents of basilar artery and aorta from rabbits with alloxan-induced diabetes. *Can. J. Physiol. Pharmacol.*, **68**, 882-888.
- ADEAGBO, A.S.O. & TRIGGLE, C.R. (1993). Varying extracellular $[K^+]$: a functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. *J. Cardiovasc. Pharmacol.*, **21**, 423-429.

- AIELLO, L.P., GARDNER, T.W., KING, G.L., BLANKENSHIP, G., CAVALLERANO, J.D., FERRIS, F.L. 3rd & KLEIN, R. (1998). Diabetic Retinopathy. *Diabetes Care*, **21**, 143-156.
- ALBARWANI, S., HEINERT, G., TURNER, J.L. & KOZLOWSKI, R.Z. (1995). Differential K⁺ channel distribution in smooth muscle cells isolated from the pulmonary artery tree of the rat. *Biochem. Biophys. Res. Commun.*, **208**, 183-189.
- ALEXANDER, W.D. & OAKE, R.J. (1977). The effect of insulin on vascular reactivity to norepinephrine. *Diabetes*, **26**, 611-614.
- AMERICAN DIABETES ASSOCIATION (2002a). Diabetic Retinopathy (Position Statement). *Diabetes Care*, **25** (Suppl 1), S90-S93.
- AMERICAN DIABETES ASSOCIATION (2002b). Diabetic Nephropathy (Position Statement). *Diabetes Care*, **25** (Suppl 1), S85-S89.
- ANDERSON, E.A., HOFFMAN, R.P., BOLON, T.W., SINKEY, C.A. & MARK, A.L. (1991). Hyperinsulinaemia produces both sympathetic neural activation and vasodilation in normal humans. *J. Clin. Invest.*, **87**, 2246-2252.
- ANGUS, J.A., JENNINGS, G.L. & SUDHIR, K. (1992). Enhanced contraction to noradrenaline, serotonin and nerve stimulation but normal endothelium-derived relaxing factor response in skin small arteries in human primary hypertension. *Clin. Exp. Pharm. Phys.*, **19** (Suppl. 19), 39-47.
- ATKINSON, M.A. & EISENBARTH, G.S. (2000). Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet*, **358**, 221-229.
- AUCH-SCHWELK, W., KATUSIC, Z.S. & VANHOUTTE, P.M. (1990). Thromboxane A₂ receptor antagonists inhibit endothelium-dependent contractions. *Hypertension*, **15**, 699-703.

- AVOGARO, A., PIARULLI, F., VALERIO, A., MIOLA, M., CALVERI, M., PAVAN, P., VICINI, P., COBELLI, C., TIENGO, A., CALO, L. & DEL PRATO, S. (1997). Forearm nitric oxide balance, vascular relaxation, and glucose metabolism in NIDDM patients. *Diabetes*, **46**, 1040-1046.
- AWAZU, M., PARKER, R.E., HARVIE, B.R., ICHIKAWA, I. & KON, V. (1991). Down regulation of endothelin-1 receptors by protein kinase C in streptozotocin diabetic rats. *J. Cardiovasc. Pharmacol.*, **17** (suppl 7), S500-S502.
- BADIMON, J.J., FUSTER, V., CHESEBRO, J.H. & BADIMON, L. (1993). Coronary atherosclerosis, a multifactorial disease. *Circ.*, **87** (Suppl. II), II-3-II-16.
- BELL, R.H. & HYE, R.J. (1983). Animal models of diabetes mellitus: physiology and pathology. *J. Surg. Res.*, **35**, 433-460.
- BELLONI, P.N., CARNET, D.H. & NICHOLSON, G.L. (1992) Organ-derived microvessel endothelial cells exhibit differential responsiveness to thrombin and other growth factors. *Microvasc. Res.*, **43**, 20-45.
- BERMAN, R.S. & GRIFFITH, T.M. (1997). Differential actions of charybdotoxin on central and daughter branch arteries of the rabbit isolated ear. *Br. J. Pharmacol.*, **120**, 639-646.
- BEVAN, J.A. & OSHER, J.V. (1972). A direct method for recording tension changes in the wall of small blood vessels *in vitro*. *Agents Actions*, **2**, 257-260.
- BOOLLEL, M. & TOOKE, J.E. (1990). The skin hyperaemic response to local injection of substance P and capsaicin in diabetes mellitus. *Diab. Med.*, **7**, 898-901.

- BOLTON, T.B., LANG, R.J. & TAKEWAKI, T. (1984). Mechanism of action of noradrenaline and carbachol on smooth muscle guinea-pig anterior mesenteric artery. *J. Physiol.*, **351**, 549-572.
- BOSNJAK, Z.J. (1993). Ion channels in vascular smooth muscle. *Anesthesiology*, **79**, 1392-1401.
- BRANDES, R.P., BEHRA, A., LEBHERZ, C., BÖGER, R.H., BODE- BÖGER, S.M., PHIVTHONG-HGAM, L. & MÜGGE, A. (1997). N^G-nitro-L-arginine- and indomethacin-resistant endothelium-dependent relaxation in the rabbit renal artery: effect of hypercholesterolaemia. *Atherosclerosis*, **135**, 49-55.
- BRAVERMAN, J.M. SUBLEY, I.M. & KEH, A. (1990). Ultrastructural analysis of the endothelial-pericyte relationship in diabetic cutaneous vessels. *J. Invest. Dermatol.*, **95**, 147-153.
- BRAYDEN, J.E. (1990). Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilation. *Am. J. Physiol.*, **259**, H668-H673.
- BROWNLEE, M., CERAMI, A. & VLISSARA, H. (1988). Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N. Eng. J. Med.*, **318**, 1315-1321.
- BUCALA, R., TRACEY, K.J. & CERAMI, A. (1991). Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J. Clin. Invest.*, **87**, 432-438.
- BUCKLEY, C.H., HADDOKE, P.W.F., HENRY, E. & O'BRIEN, C.J. (2002). Selective impairment of endothelium-mediated inhibition of vasoconstriction in patients with normal pressure glaucoma. *Br. J. Ophthalmol.* **86**, 227-232.

- BUCKLEY, C.H., MCINTYRE, C.A. & HADOKE, P.W.F. (1999). Heterogeneity of endothelial cell function in vessels isolated from human sub-cutaneous fat. *Br. J. Pharmacol.*, **128**, (Proc Suppl), P263
- BUSSE, R., POHL, U., MULSCH, A & BASSENGE, F. (1989). Modulation of the vasodilator action of SIN-1 by the endothelium. *J. Cardiovasc. Pharmacol.*, **14**, S81-S85.
- BUUS, N.H., VAN BAVEL, E. & MULVANY, M.J. (1994). Differences in sensitivity of rat mesenteric small arteries to agonists when studied as ring preparations or as cannulated preparations. *Br. J. Pharmacol.*, **112**, 579-587.
- CALVER, A., COLLIER, J. & VALLANCE, P. (1992). Inhibition and stimulation of nitric oxide synthesis in the human forearm arterial bed of patients with insulin-dependent diabetes. *J. Clin. Invest.*, **90**, 2548-2554.
- CAMBRIDGE, D., WHITING, M.V., BUTTERFIELD, L.J. & MARSTON, C. (1995). Vascular 5-HT₁-like receptors mediating vasoconstriction and vasodilatation: their characterisation and distribution in the isolated canine cardiovascular system. *Br. J. Pharmacol.*, **114**, 961-968.
- CAMERON, N.E. & COTTER, M.A. (1992). Impaired contraction and relaxation in aorta from streptozotocin-diabetic rats: role of polyol pathway. *Diabetologia*, **35**, 1011-1019.
- CARRIER, O., MURPHY, J.C. & TENNER, T.E. (1973). Effect of cold storage on calcium-related responses and electrolyte content of rabbit aortic strips. *Eur. J. Pharmacol.*, **24**, 225-233.
- CELERMAJER, D.S. (1998). Testing endothelial function using ultrasound. *J. Cardiovasc. Pharmacol.*, **32** (Suppl. 3), S29-S32.

- CHAKRAVARTHY, U., MCGINTY, A., MCKILLOP, J., ANDERSON, P., ARCHER, D.B. & TRIMBLE, E.R. (1994). Altered endothelin-1 induced contraction and second messenger generation in bovine retinal microvascular pericytes cultures in high glucose medium. *Diabetologia*, **37**, 36-42.
- CHAN, N.N., VALLANCE, P. & COLHOUN, H.M. (2000). Nitric oxide and vascular responses in type I diabetes. *Diabetologia*, **43**, 137-147.
- CHAYTOR, A.T., EVANS, W.H. & GRIFFITH, T.M. (1998). Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. *J. Physiol.*, **508**, 561-573.
- CHIBBER, R., MOLINATTI, P.A., WONG, J.S., MIRLEES, D. & KOHNER, E.M. (1994). The effect of aminoguanidine and tolrestat on glucose toxicity in bovine retinal capillary pericytes. *Diabetes*, **43**, 758-763.
- CHOWIENCZYK, P.J., COCKCROFT, J.R. & RITTER, J.M. (1993). Differential inhibition by N^G-monomethyl-L-arginine of vasodilator effects of acetylcholine and methacholine in human forearm vasculature. *Br. J. Pharmacol.*, **110**, 736-738.
- CHOWIENCZYK, P.J., COCKCROFT, J.R. & RITTER, J.M. (1994). Blood flow responses to intra-arterial acetylcholine in man: effects of basal blood flow and conduit vessel length. *Clin. Sci.*, **87**, 45-51.
- CIPOLLA, M.J., HARKER, C.T. & PORTER, J.M. (1996). Endothelial function and adrenergic reactivity in human type-II diabetic resistance arteries. *J. Vasc. Surg.*, **23**, 940-949.
- CLOZEL, M. GRAY, G.A., BREU, V., LOTTLE, B-M & OSTERWALDER, R. (1992). The endothelin ET_B receptor mediates both vasodilation and vasoconstriction *in vivo*. *Biochem. Biophys. Res. Commun.*, **186**, 867-873.

- CLOZEL, M., LOFFLER, B-M., BREU, U., HILFIGER, L., MAIRE, J-P & BITSCHA, B. (1993). Downregulation of endothelin receptors by autocrine production of endothelin-1. *Am. J. Physiol.*, **265**, C188-C192.
- COCKCROFT, J.R., WHITE, D.J., PAGE, S.R. & GAZIS, A.G. (1998). Preserved endothelium-dependent vasodilation in subjects with uncomplicated Type 2 diabetes. *Diabetologia*, **41** (Suppl. 1), A1-A380,1206.
- COHEN, R.A. & VANHOUTTE, P.M. (1995). Endothelium-dependent hyperpolarization. Beyond nitric oxide and cyclic GMP. *Circulation*, **92**, 3337-3349.
- COHEN, R.A. (1993). Pathways controlling healthy and diseased arterial smooth muscle. *Am. J. Cardiol.*, **72**, 39C-47C.
- COLLINS, P.B., GRIFFITH, T.M., HENDERSON, A.H. & LEWIS, M.J. (1986) Endothelium-derived relaxing factor alters calcium fluxes in rabbit aorta: a cyclic guanosine monophosphate-mediated effect. *J. Physiol.*, **381**, 427-437.
- CORBETT, J.A., TILTON, R.G., CHANG, K., HASAN, K.S., IDO, Y., WANG, H., SWEETLAND, M.A.M LANCASTER, J.R., WILLIAMSON, J.R. & MCDANIEL, M.L. (1992). Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. *Diabetes*, **41**, 552-556.
- CORRIU, C., FELETOU, M., CANET, E. & VANHOUTTE, P.M. (1996). Endothelium-derived factors and hyperpolarization of the carotid artery of the guinea-pig. *Br. J. Pharmacol.*, **119**, 959-964.
- COSENTINO, F., HISHIKAWA, K., KATUSIC, Z.S. & LUSCHER, T.F. (1997). High glucose increase nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. *Circulation*, **96**, 25-28.

- COWAN, C.L., PALACINO, J.J., NAJIBI, S. & COHEN, R.A. (1993). Potassium-channel-mediated relaxation to acetylcholine in rabbit arteries. *J. Pharmacol. Exp. Ther.*, **266**, 1482-1489.
- CREAGER, M.A., LIANG, C.S. & COFFMAN, J.D. (1985). Beta adrenergic-mediated vasodilator response to insulin in the human forearm. *J. Pharmacol. Exper. Ther.*, **235**, 709-714.
- DABROWSKA, R., SHERRY, J.M.F., AROMATORIO, D.K. & HARTSHORNE, D.J. (1978). Modulator protein as a component of the myosin light chain kinase from chicken gizzard. *Biochemistry*, **17**, 253-258.
- DE TEJADA, I.S., GOLDSTEIN, I., AZADZOI, K., KRANE, R.J. & COHEN, R.A. (1989). Impaired neurogenic and endothelium-mediated relaxation of penile smooth muscle from diabetic men with impotence. *N. Engl. J. Med.*, **320**, 1025-1030.
- DEMEY, F.G. & GRAY, S.D. (1985). Endothelium-dependent reactivity in resistance vessels. *Prog. Appl. Microcirc.*, **8**, 181-187.
- DENG, L-Y., LI, J-S. & SCHIFFRIN, L. (1995). Endothelium-dependent relaxation of small arteries from essential hypertensive patients: mechanisms and comparison with normotensive subjects and with responses of vessels from spontaneously hypertensive rats. *Clin. Sci.*, **88**, 611-622.
- DENUCCI, G., THOMAS, R., D'ORLEANS-JUSTE, P., ANTUNES, E., WALDER, C., WARNER, T.D. & VANE, J.R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. USA*, **85**, 9797-9800.

- DIABETES CONTROL AND COMPLICATIONS TRIAL GROUP (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med.*, **329**, 977-986.
- DIEDERICH, D., SKOPEC, J., DIEDERICH, A. & DAI, F-X. (1994). Endothelial dysfunction in mesenteric resistance arteries of diabetic rats: role of free radicals. *Am. J. Physiol.*, **266**, H1153-H1161.
- DOHI, Y., THIEL, M.A., BUHLER, F.R. & LUSCHER, T.F. (1990). Activation of endothelial L-arginine pathway in resistance arteries. *Hypertension*, **15**, 170-179.
- DOUGHTY, J.M., BOYLE, J.P. & LANGTON, P. (2000). Potassium does not mimic EDHF in rat mesenteric arteries. *Br. J. Pharmacol.*, **130**, 1174-1182.
- DUFFY, S.J., TRAN, B.T., NEW, G., TUDBALL, R.N., ESLER, M.D., HARPER, R.W. & MEREDITH, I.T. (1998). Continuous release of vasodilator prostanoids contributes to regulation of resting forearm blood flow in humans. *Am. J. Physiol.*, **274**, H283-H289.
- DURANTE, W., SEN, A.K. & SUNAHARA, F.A. (1988). Impairment of endothelium-dependent relaxation in aortae from spontaneously diabetic rats. *Br. J. Pharmacol.*, **94**, 463-468.
- EBERL, T., SCHMID, T., WODLINGER, R., HENGSTER, P., HEROLD, M., WEISS, H., OBERHUBER, G., KRAUSLER, R. & MARGREITER, R. (1993). Which organ preservation solution best protects vascular endothelium? *Transplant. Proceed.*, **25**, 3019.

- EDWARDS, G., DORA, K.A., GARDENER, M.J., GARLAND, C.J. & WESTON, A.H. (1998). K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature*, **396**, 269-272.
- EHRINGER, W.D., SU, S., CHIANG, B., STILLWELL, W. & CHIEN, S.F. (2002). Destabilizing effects of fructose-1,6-biphosphate on membrane bilayers. *Lipids*, **37**, 885-892.
- ELLIOTT, T.G., COCKCROFT, J.R., GROOP, P.-H., VIBERTI, G.C. & RITTER, J.M. (1993). Inhibition of nitric oxide synthesis in forearm vasculature of insulin-dependent diabetic patients: blunted vasoconstriction in patients with microalbuminuria. *Clin. Sci.*, **85**, 687-693.
- ENDERLE, M.D., BENDA, N., SCHMUELLING, R.M., HAERING, H.U. & PFOHL, M. (1998). Preserved endothelial function in IDDM patients, but not in NIDDM patients, compared with healthy subjects. *Diabetes Care*, **21**, 271-277.
- EURODIAB IDDM COMPLICATIONS STUDY GROUP. (1994). Microvascular and acute complications in IDDM patients: the Eurodiab IDDM Complications Study. *Diabetologia*, **36**, 454-459.
- FALLOON, B.J., BUND, S.J., TULIP, J.R. & HEAGERTY, A.M. (1993). *In vitro* perfusion studies of resistance artery function in genetic hypertension. *Hypertension Dallas*, **22**, 486-495.
- FALLOON, B.J., STEPHENS, N., TULIP, J.R. & HEAGERTY, A.M. (1995). Comparison of small artery sensitivity and morphology in pressurized and wire-mounted preparations. *Am. J. Physiol.*, **268**, H670-H678.

- FEELISCH, M. & NOACK, E.A. (1987). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.*, **139**, 19-30.
- FEELISCH, M. (1991). The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J. Cardiovasc. Pharmacol.*, **17**, S25-S33.
- FELETOU, M. & VANHOUTTE, P.M. (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br. J. Pharmacol.*, **93**, 515-524.
- FELETOU, M. & VANHOUTTE, P.M. (1997). Endothelium-derived hyperpolarizing factor. *Clin. Exp. Pharmacol. Physiol.*, **23**, 1082-1090.
- FELETOU, M. & VANHOUTTE, P.M. (1997). EDHF exists. *Br. J. Pharmacol.*, **122**, (Suppl.), U142.
- FILO, R.S., BOHR, D.F. & RÜEGG, J.C. (1965). Glycerinated skeletal and smooth muscle: calcium and magnesium dependence. *Science (Washington D.C.)*, **147**, 1581-1583.
- FLANDERS, S., HARDY, K.J. & LEW, M.J. (1996). Effect of cold storage in University of Wisconsin solution on the responses of porcine hepatic arteries to 5-hydroxytryptamine and bradykinin *in vitro*. *Br. J. Pharmacol.*, **117**, 142-146.
- FORSTERMANN, U., HERTTING, G. & NEUFANG, B. (1986). The role of endothelial and non-endothelial prostaglandins in the relaxation of isolated blood vessels of the rabbit induced by acetylcholine and bradykinin. *Br. J. Pharmacol.*, **87**, 521-532.

- FORSTERMANN, U., SCHMIDT, H.H.H.W., POLLOCK, J.S., SHENG, H., MITCHELL, J.A., WARNER, T.D., NAKANE, M. & MURAD, F. (1991). Isoforms of nitric oxide synthase. Characterisation and purification from different cell types. *Biochem. Pharmacol.*, **42**, 1849-1857.
- FRIER, B.M., TRUSWELL, A.S., SHEPHERD, J., DELOOY, A. & JING, R. (1999). Diabetes mellitus and nutritional and metabolic disorders. In Davidson's Principles and Practice of Medicine, Editors Haslett, C., Chilvers, E.R., Hunter, J.A. & Boon, N.A. Chp 7, p471-542, 18th edition, Churchill Livingstone, Edinburgh.
- FULTON, D.J.R., HODGSON, W.C., SIKARSKI, B.W. & KING, R.G. (1991). Attenuated responses to endothelin-1, KCl and CaCl₂, but not noradrenaline, of aorta from rats with streptozotocin-induced diabetes mellitus. *Br. J. Pharmacol.*, **104**, 928-932.
- FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373-376.
- FURCHGOTT, R.F. (1984). The role of the endothelium in the responses of vascular smooth muscle to drugs. *Annu. Rev. Pharmacol. Toxicol.*, **24**, 175-197.
- FURCHGOTT, R.F., CARVALHO, M.H., KHAN, M.T. & MATSUNAGA, K. (1987). Evidence for endothelium-dependent vasodilation of resistance vessels by acetylcholine. *Blood Vessels* **24**, 145-149.
- FURNESS, J.B. & MARSHALL, J.M. (1974). Correlation of the directly observed responses of mesenteric vessels of the rat to nerve stimulation and noradrenaline with the distribution of adrenergic nerves. *J. Physiol.*, **239**, 75-88.

- GARLAND, C.J. & MCPHERSON, G.A. (1992). Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *Br. J. Pharmacol.*, **105**, 429-435.
- GIUGLIANO, D., CERIELLO, A. & PAOLISSO, G. (1996). Oxidative stress and diabetic vascular complications. *Diabetes Care*, **19**, 257-267.
- GUAZZAROTTI, L., BARTOLOTTA, E. & CHIARELLI, F. (1999). Maturity-onset diabetes of the young (MODY): a new challenge for pediatric diabetologists. *J. Pediatr. Endocrinol. Metab.*, **12**, 487-497.
- GURNEY, A.M. (1994). Mechanisms of drug-induced vasodilation. *J. Pharm. Pharmacol.*, **46**, 242-251.
- GUSTAFSSON, H. & NILSSON, H. (1990). Endothelium-independent potentiation by neuropeptide Y of vasoconstrictor responses in isolated arteries from rat and rabbit. *Acta Physiol. Scand.*, **138**, 503-507.
- HADOKE, P.W.F., MCINTYRE, C.A., BUCKLEY, C.H., JONES, G.C., ELLIOT, A.I., MCKNIGHT, J., WILLIAMS, B.C. & WALKER, B.R. (1999). Endothelium-dependent potassium chloride-mediated relaxation of human resistance arteries. *Br. J. Pharmacol.*, **128**, Proc. Suppl., 264P.
- HAEFLIGER, I.O., FLAMMER, J. & LUSCHER, T.F. (1993) Heterogeneity of endothelium-dependent regulation in ophthalmic and ciliary arteries. *Invest. Ophthalmol. Vis. Sci.*, **34**, 1722-1730.
- HALKIN, A., BANJAMIN, N., DOKTOR, H.S., TODD, S.D., VIBERTI, G. & RITTER, J.M. (1991). Vascular responsiveness and cation exchange in insulin-dependent diabetes. *Clin. Sci.*, **81**, 223-232.

- HAN, S-Z., OUCHI, Y., KARAKI, H. & ORIMO, H. (1995). Inhibitory effects of insulin on cytosolic Ca^{2+} level and contraction in the rat aorta. Endothelium-dependent and -independent mechanisms. *Circ. Res.*, **77**, 673-678.
- HARDER, D.R. (1987). Pressure-induced myogenic activation of cat cerebral arteries is dependent on an intact endothelium. *Circ. Res.*, **60**, 102-107.
- HARRIS, K.H. & MACLEOD, K.M. (1988). Influence of the endothelium on contractile responses of arteries from diabetic rats. *Eur. J. Pharmacol.*, **153**, 55-64.
- HATTERSLEY, A.T. (1998). Maturity-onset diabetes of the young: clinical heterogeneity explained by genetic heterogeneity. *Diabet. Med.*, **15**, 15-24.
- HATTORI, Y., KASAI, K., NAKAMURA, T., EMOTO, T. & SHIMADA, S. (1991). Effect of glucose and insulin on immunoreactive endothelin-1 release from cultured porcine aortic endothelial cells. *Metabolism*, **40**, 165-169.
- HATTORI, Y., KAWASAKI, H., ABE, K. & KANNO, M. (1991). Superoxide dismutase recovers altered endothelium-dependent relaxation in diabetic rat aorta. *Am. J. Physiol.*, **261** (Heart Circ. Physiol., 30), H1086-H1094.
- HAWTHORNE, G.C., BARTLETT, K., HETHERINGTON, C.S. & ALBERTI, K.G.M.M. (1989). The effect of high glucose on polyol pathway activity and myoinositol metabolism in cultured human endothelial cells. *Diabetologia*, **32**, 163-166.
- HAYNES, W.G. & WEBB, D.J. (1993). The endothelin family of peptides: local hormones with diverse roles in health and disease? *Clin. Sci.*, **84**, 485-500.
- HAYNES, W.G. & WEBB, D.J. (1998). Endothelin as a regulator of cardiovascular function in health and disease. *J. Hypertens.*, **16**, 1081-1098.

- HEAD, J. & FULLER, J.H. (1990). International variations in mortality among diabetic patients: the WHO Multinational Study of Vascular Disease in Diabetics. *Diabetologia*, **33**, 477-481.
- HECKER, M., BARA, A.T., BAUERSACHS, J. & BUSSE, R. (1994). Characterisation of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *J. Physiol.*, **481**, 407-414.
- HERRMANN-FRANK, A., DARLING, E. & MEISSNER, G. (1991). Functional characterisation of the Ca^{2+} -gated Ca^{2+} release channel of vascular smooth muscle sarcoplasmic reticulum. *Pfluegers Arch.*, **418**, 353-359.
- HEYGATE, K.M., BENNETT, M.A., WATT, P.A.C. & MCNALLY, P.G. (1993). The effect of glucose concentration on endothelium-dependent relaxation in resistance vessels. *Diab. Med.*, **10** (suppl 1), S33.
- HEYGATE, K.M., LAWRENCE, I.G., BENNETT, M.A. & THURSTON, H. (1995). Impaired endothelium-dependent relaxation in isolated resistance arteries of spontaneously diabetic rats. *Br. J. Pharmacol.*, **116**, 113-117.
- HILLIER, C., MCGEE, A. & McGRATH, J.C. (1998). Bradykinin-induced vasorelaxation of human subcutaneous resistance arteries does not involve prostacyclin.
- HODGSON, W.C. & KING, R.G. (1992). Effects of glucose, insulin or aldose reductase inhibition on responses to endothelin-1 of aortic rings from streptozotocin-induced diabetic rats. *Br. J. Pharmacol.*, **106**, 644-649.
- HOPFNER, R.L. & GOPALAKRISHNAN, V. (1999). Endothelin: emerging role in diabetic vascular complications. *Diabetologia*, **42**, 1383-1394.

- HOPFNER, R.L., MCNEILL, J.R. & GOPALAKRISHNAN, V. (1999). Endothelin plasma levels and endothelin-1 evoked vascular responses at different temporal stages of diabetes in streptozotocin diabetic rats. *Eur. J. Pharmacol.*, **374**, 221-227.
- HOSTETTER, T.H., RENNKE, H.G. & BRENNER, B.M. (1982). The case for intrarenal hypertension in the initiation and progression of diabetic and other glomerulopathies. *Am. J. Med.*, **72**, 375-380.
- HOUBEN, A.J.H.M., SCHAPER, N.C., DE HAAN, C.H.A., HUVERS, F.C., SLAAF, D.W., DE LEEUW, P.W. & NIEUWENHUIJZEN KRUSEMAN, A.C. (1996). Local 24-h hyperglycaemia does not affect endothelium-dependent or – independent vasoreactivity in humans. *Am. J. Physiol.*, **270**, H2014-H2020.
- HU, R-M., LEVIN, E.R., PEDRAM, A. & FRANK, H.J.L. (1993). Insulin stimulates production and secretion of endothelin from bovine endothelial cells. *Diabetes*, **42**, 351-355.
- IGNARRO, L.J., HARBISON, R.G., WOOD, K.S. & KADOWITZ, P.J. (1986) Dissimilarities between methylene blue and cyanide on relaxation and cyclic GMP formation in endothelium-intact intrapulmonary artery caused by nitric oxide-containing vasodilators and acetylcholine. *J. Pharmacol. Exp. Ther.*, **236**, 30-36.
- IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. USA*, **84**, 9265-9269.

- INOUE, A., YANAGISAWA, M., KIMURA, S. ET AL. (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci. USA*, **86**, 2863-2867.
- JAAP, A.J. & TOOKE, J.E. (1995) Pathogenesis of microvascular disease I non-insulin-dependent diabetes. *Clin. Sci.*, **89**, 3-12.
- JAAP, A.J., SHORE, A.C. & TOOKE, J.E. (1996). Differences in microvascular fluid permeability between long duration type-I diabetic patients with and without significant microangiopathy. *Clin. Sci.*, **90**, 113-117.
- JACKSON, R.M., CHANDLER, D.B. & FULMER, J.D. (1986). Production of arachidonic acid metabolites by endothelial cells in hyperoxia. *J. Appl. Physiol.*, **61**, 584-591.
- JAMES, M.A., WATT, P.A.C., POTTER, J.F., THURSTON, H. & SWALES, J.D. (1997). Endothelial function in subcutaneous resistance arteries from elderly hypertensive and normotensive subjects. *Clin. Sci.*, **92**, 139-145.
- JARRETT, R.J. (1989). Cardiovascular disease and hypertension in diabetes mellitus. *Diabetes Metab. Rev.*, **5**, 547-558.
- JOHNSTONE, M.T., CREAGER, S.,J., SCALES, K.M., CUSCO, J.A., LEE, B.K. & CREAGER, M.A. (1993). Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *Circulation*, **88**, 2510-2516.
- KAGOTA, S., TAMASHIRO, A., YAMAGUCHI, Y., NAKAMURA, K. & KUNITOMO, M. (1999). Excessive salt or cholesterol intake alters the balance among endothelium-derived factors released from renal arteries in spontaneously hypertensive rats. *J. Cardiovasc. Pharmacol.*, **34**, 533-539.

- KANEKO, K. & SUNANO, S. (1993). Involvement of α -adrenoceptors in the endothelium-dependent depression of noradrenaline-induced contraction in rat aorta. *Eur. J. Pharmacol.*, **240**, 195-200.
- KATUSIC, Z.X. (2002). Back to the salt mines- endothelial dysfunction in hypertension and compensatory role of endothelium-derived hyperpolarizing factor (EDHF). *J. Physiol.*, **543**, 255-260.
- KERN, T.S. & ENGERMAN, R.L. (1982). Immunohistochemical distribution of aldose reductase. *Histochem. J.*, **14**, 507-515.
- KOHNER, E.M., PATEL, V. & RASSAM, S.M.B. (1995). Role of blood flow and impaired autoregulation in the pathogenesis of diabetic retinopathy. *Diabetes*, **44**, 603-607.
- KRISTEK, F., TOROK, J. & SIKVLOVA, J. (1993). Morphological and functional alterations in endothelium, smooth muscle and nerve fibers in rabbit aorta after storage at 4°C. *Cryobiology*, **30**, 376-385.
- KU, D.D., WILLIS, W.L. & CAULFIELD, J.B. (1990). Retention of endothelium-dependent vasodilatory responses in canine coronary arteries following cryopreservation. *Cryobiology*, **27**, 511-520.
- LACY, P.S., PILKINGTON, G., HANVESAKUI, R., FISH, H.J., BOYLE, J.P. & THURSTON, H. (2000). Evidence against potassium as an endothelium-derived hyperpolarizing factor in rat mesenteric small arteries. *Br. J. Pharmacol.*, **129**, 605-611.
- LAMANTAGNE, D., KANIG, A., BASSENGE, E. ET AL., (1992). Prostacyclin and nitric oxide contribute to the vasodilator action of acetylcholine and

- bradykinin in the intact rabbit coronary bed. *J. Cardiovasc. Pharmacol.*, **20**, 652-657.
- LAMBERT, J., AARSEN, M., DONKER, A.J. & STEHOUWER, C.D. (1996). Endothelium-dependent and -independent vasodilation of large arteries in normoalbuminuric insulin-dependent diabetes mellitus. *Arterioscler. Thromb. Vasc. Biol.*, **16**, 705-711.
- LAMONTAGNE, D., POHL, U. & BUSSE, R. (1992). Mechanical deformation of vessel wall and shear stress determine the basal release of endothelium-derived relaxing factor in the intact rabbit coronary vascular bed. *Circ. Res.*, **70**, 123-130.
- LAWRENCE, E. & BRAIN, S.D. (1992). Altered microvascular reactivity to ET-1, ET-3 and L-N^G-nitro-arginine methyl ester in streptozotocin-induced diabetes mellitus. *Br. J. Pharmacol.*, **100**, 1035-1040.
- LAWRENCE, I.G., WATT, P.A.C., MCNALLY, P.G., BURDEN, A.C. & THURSTON, H. (1994). Resistance artery structure and function in non-insulin-dependent diabetes mellitus (NIDDM). *Diabetologia*, **37** (Suppl. 1), A22, 79.
- LEKAKIS, J., PAPAMICHAEL, C., ANASTASIOU, H. ET AL (1997). Endothelial dysfunction of conduit arteries in insulin-dependent diabetes mellitus without microalbuminuria. *Cardiovasc. Res.*, **34**, 164-168.
- MACINTYRE, D.E., PEARSON, J.D. & GORDON, J.L. (1978). Localisation and stimulation of prostacyclin production in vascular cells. *Nature, Lond.*, **271**, 549-551.
- MAKIMATTILA, S., MANTYSAARI, M., GROOP, P.H., ET AL. (1997). Hyper-reactivity to nitrovasodilators in fore-arm vasculature is related to autonomic dysfunction in insulin-dependent diabetes mellitus. *Circ.*, **95**, 618-625.

- MAKITA, Z., RADOFF, S., RAYFIEL, E.J., YANG, Z., SKOLNIK, E., DELANEY, V., FRIEDMAN, E.A., CERAMI, A. & VLASSARA, H. (1991). Advanced glycosylation end products in patients with diabetic nephropathy. *N. Eng. J. Med.*, **325**, 836-842.
- MALIK, R.A., PANIAGUA, O., SHAW, L., AUSTIN, C. & HEAGERTY, A.M. (1999). Resistance vessel function and structure in type 1 diabetes. *Diabetologia*, **42** (Suppl. 1), A75 (Abstract).
- MALIK, R.A., TESFAYE, S., THOMPSON, S.D., VEVES, A., SHARMA, A.K., BOLTON, A.J. & WARD, J.D. (1993). Endoneurial localisation of microvascular damage in human diabetic neuropathy. *Diabetologia*, **36**, 454-459.
- MANN, G.E., YUDILEVICH, D.L. & SOBREVIA, L. (2003). Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiol. Rev.*, **83**, 183-252.
- MATOBA, T., SHIMOKAWA, H., NAKASHIMA, M., HIIRAKAWA, Y., MUKAI, Y., HIRANO, K., KANAIDE, H. & TAKESHITA, A. (2000). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J. Clin. Invest.*, **106**, 1521-1530.
- MAYER, B., SCHRAMMEL, A., KLATT, P., KOESLING, D. & SCHMIDT, K. (1995). Peroxynitrite-induced accumulation of cyclic GMP in endothelial cells and stimulation of purified soluble guanylate cyclase. Dependence on glutathione and possible role of S-nitrosation. *J. Biol. Chem.*, **270**, 17355-17360.
- MCARTHY, A.L., WOOLFSON, R.G., RAJU, S.K. & POSTON, L. (1993). Abnormal endothelial cell function of resistance arteries from women with preeclampsia. *Am. J. Obstet. Gynecol.*, **168**, 1323-1330.

- MCINTYRE, C.A., ANDREWS, R.C., ELLIOT, A., GRAY, G.A., WILLIAMS, B.C., MCKNIGHT, J., WALKER, B.R. & HADOKE, P.W.F. (1998). Endothelium-derived hyperpolarising factor mediates to a large extent acetylcholine-induced relaxation of human sub-cutaneous resistance arteries. *Br. J. Pharmacol.*, **125**, Proc Suppl., P16 (Abstract).
- MCINTYRE, C.A., WILLIAMS, B.C., ANDREWS, R.C., MCKNIGHT, J.A., WALKER, B.R. & HADOKE, P.W.F. (1998). Impaired receptor-independent vasoconstriction in resistance arteries isolated from patients with non-insulin-dependent diabetes mellitus. *Diabetic Medicine*, **15**, (Suppl. 2), P6.
- MCNALLY, P.G., LAWRENCE, I.G., WATT, P.A.C., HILLIER, C., BURDEN, A.C. & THURSTON, H. (1995). The effect of insulin on the vascular reactivity of isolated resistance arteries taken from healthy volunteers. *Diabetologia*, **38**, 467-473.
- MCNALLY, P.G., WATT, P.A.C., RIMMER, T., BURDEN, A.C., HEARNshaw, J.R. & THURSTON, H. (1994). Impaired contraction and endothelium-dependent relaxation in isolated resistance vessels from patients with insulin-dependent diabetes mellitus. *Clin. Sci.*, **87**, 31-36.
- McVEIGH, G.E., BRENNAN, G.M., JOHNSTON, G.D., McDERMOTT, B.J., McGRATH, L.T., HENRY, W.R., ANDREWS, J.W. & HAYES, J.R. (1992). Impaired endothelium-dependent and independent vasodilation in patients with Type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia*, **35**, 771-776.
- MIASIRO, N. & PAIVA, A.C.M. (1990). Homologous desensitization of the effects of endothelin on rabbit aorta rings and on cultured rat aorta smooth muscle cells. *Eur. J. Pharmacol.*, **179**, 151-158.

- MISSIAEN, L., DE SMEDT, H., DROOGMANS, G., HIMPENS, B. & CASTEELS, R. (1992). Calcium ion homeostasis in smooth muscle. *Pharmacol. Ther.*, **56**, 191-231.
- MONCADA, S. & HIGGS, EA. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.*, **329**, 2002-2012.
- MONCADA, S. & VANE, J.R. (1979). Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A₂ and prostacyclin. *Pharmacol. Rev.*, **30**, 293-331.
- MONCADA, S. (1982). Biological importance of prostacyclin. *Br. J. Pharmacol.*, **76**, 3-31.
- MONCADA, S., GRYGLEWSKI, R.J., BUNTING, S. & VANE, J.R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature, Lond*, **263**, 663-665.
- MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109-142.
- MONCADA, S., REES, D.D., SCHULZ, R. & PALMER, R.M.J. (1991). Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis *in vivo*. *Proc. Natl. Acad. Sci. USA*, **88**, 2166-2170.
- MORABITO, E., CORSICO, N. & MARTELLI, E.A., (1995). Endothelins urinary excretion is increased in spontaneously diabetic rats: BB/ BB. *Pharm. Lett.*, **56**, PL13-18.
- MORO, M.A., DARLEY-USMAR, V.M., GOODWIN, D.A., READ, N.G., ZAMORA-PINO, R., FEELISCH, M., RADOMSKI, M.W. & MONCADA, S.

- (1994). Paradoxical fate and biological action of peroxynitrite on human platelets. *Proc. Natl. Acad. Sci. USA*, **91**, 6702-6706.
- MORRIS, S.J., SHORE, A.C. & TOOKE, J.E. (1995). Responses of the skin microcirculation to acetylcholine and sodium nitroprusside in patients with NIDDM. *Diabetologia*, **38**, 1337-1344.
- MORRISH, N.J., STEVENS, L.K., FULLER, J.H., JARRETT, R.J. & KEEN, H. (1991). Risk factors for macrovascular disease in diabetes mellitus: the London follow-up to the WHO Multinational Study of Vascular Disease in Diabetics. *Diabetologia*, **34**, 590-594.
- MULLER-SCHWIENITZER, E., TAPPARELLI, C. & VICTORZON, M. (1986). Functional studies on human veins after storage at -190°C. *Br. J. Pharmacol.*, **88**, 685-687.
- MULSCH, A. & BUSSE, R. (1990). N^G-nitro-L-arginine (N^S-[imino-(nitroamino)methyl]-L-ornithine) impairs endothelium-dependent dilatations by inhibiting cytosolic nitric oxide synthesis from L-arginine. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **341**, 143-147.
- MULVANY, M.J. & HALPERN, W. (1976). Mechanical properties of vascular smooth muscle cells *in situ*. *Nature Lond.*, **260**, 617-619.
- MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.*, **41**, 19-26.
- MULVANY, M.J. & NYBORG, N.C.B. (1980). An increased calcium sensitivity of mesenteric resistance vessels in young and adult spontaneously hypertensive rats. *Br. J. Pharmacol.*, **71**, 585-596.

- MULVANY, M.J. (1983). Do resistance vessel abnormalities contribute to the elevated blood pressure of spontaneously hypertensive rats? A review of some of the evidence. *Blood Vessels*, **20**, 1-22.
- MULVANY, M.J., HANSEN, P.K. & AALKJAER, C. (1978). Direct evidence that the greater contractility of resistance vessels in spontaneously hypertensive rats is associated with a narrowed lumen, a thickened media, and an increased number of smooth muscle cell layers. *Circ. Res.*, **43**, 854-864.
- MURPHY, M.E. & BRAYDEN, J.E. (1995). Apamin-sensitive K^+ channels mediate an endothelium-dependent hyperpolarization in rabbit mesenteric arteries. *J. Physiol.*, **489**, 723-734.
- NAGAO, T. & VANHOUTTE, P.M. (1992). Hyperpolarization as a mechanism for endothelium-dependent relaxations in the porcine coronary artery. *J. Physiol.*, **445**, 355-367.
- NAGAO, T., ILLIANO, S. & VANHOUTTE, P.M. (1992). Heterogeneous distribution of endothelium-dependent relaxations resistant to N^G -nitro-L-arginine in rats. *Am. J. Physiol.*, **263**, H1090-H1094.
- NAKASHIMA, M., MOMBOULI, J-V., TAYLOR, A.A. & VANHOUTTE, P.M. (1993). Endothelium-dependent hyperpolarization caused by bradykinin in human coronary arteries. *J. Clin. Invest.*, **92**, 2867-2871.
- NAKHOODA, I., LIKE, A.A., CHAPPEL, C.I., MURRAY, F.T. & MARLISS, E.B. (1977). The spontaneously diabetic Wistar rat: metabolic and morphologic studies. *Diabetes*, **26**, 100-112.

- NELSON, M.T., PATLAK, J.B., WORLEY, J.F. & STANDEN, N.B. (1990). Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am. J. Physiol.*, **259**, C3-C18.
- NILSSON, H. & SJÖBLOM, N. (1985). Distension-dependent changes in noradrenaline sensitivity in small arteries from the rat. *Acta Physiol. Scand.*, **125**, 429-435.
- NISHIDA, K., HARRISON, D.G., NAVAS, J.P., FISHER, A.A., DOCKERY, S.P., UEMATSU, M., NEREM, R.M., ALEXANDER, R.W. & MURPHY, T.J. (1992). Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J. Clin. Invest.*, **90**, 2092-2096.
- NUGENT, A.G., MCGURK, C., HAYES, J.R. & JOHNSTON, G.D. (1996). Impaired vasoconstriction to endothelin-1 in patients with NIDDM. *Diabetes*, **45**, 105-107.
- OHLMANN, P., MARTINEZ, M.C., SCHNEIDER, F., STOCLET, J.C. & ANDRIANTSITOHAINA, R. (1997). Characterization of endothelium-derived relaxing factors released by bradykinin in human resistance arteries. *Br. J. Pharmacol.*, **121**, 657-664.
- OLIVER, F.J., DELARUBIA, G., FEENER, E.P., LEE, M-E., LOEKEN, M.R., SHIBA, T., QUERTERMOUS, T. & KING, G.L. (1991). Stimulation of endothelin-1 gene expression by insulin in endothelial cells. *J. Biol. Chem.*, **266**, 23251-23256.
- OLMOS, P., HERN, R.A., HEATON, D.A., MILLWARD, B.A., RISLEY, D., PYKE, D.A. & LESLIE, R.D.G. (1988). The significance of the concordance rate

- for type 1 (insulin-dependent) diabetes in identical twins. *Diabetologia*, **31**, 741-750.
- OSOL, G., CIPOLLA, M. & KNUTSON, S. (1989). A new method for mechanically denuding the endothelium of small (50-150µm) arteries with a human hair. *Blood Vessels*, **26**, 320-324.
- OZTURK, Y., ALTAN, V.M. & YILDIZOGLU-ARI, N. (1996). Effects of experimental diabetes and insulin on smooth muscle functions. *Pharmacol. Rev.*, **48**, 69-112.
- PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664-666.
- PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature (Lond.)*, **327**, 524-526.
- PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524-526.
- PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988b). L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251-1256.
- PARVING, H-H., VIBERTI, G.C., KEEN, H., CHRISTIANSEN, J.S. & LASSEN, N.A. (1983). Hemodynamic factors in the genesis of diabetic microangiopathy. *Metabolism*, **32**, 943-949.

- PASCOAL, I.F. & UMANS, J.G. (1996). Effect of pregnancy on mechanisms of relaxation in human omental microvessels. *Hypertension*, **28**, 183-187.
- PETERSSON, J., ZYGMUNT, P.M. & HOGESTATT, E.D. (1997). Characterization of the potassium channels involved in EDHF-mediated relaxation in cerebral arteries. *Br. J. Pharmacol.*, **120**, 1344-1350.
- PETERSSON, J., ZYGMUNT, P.M., BRANDT, L. & HOGESTATT, E.D. (1995). Substance P-induced relaxation and hyperpolarization in human cerebral arteries. *Br. J. Pharmacol.*, **115**, 889-894.
- PIEPER, G.M. (1997). Divergent actions of chronic insulin treatment *in vivo* versus acute treatment *ex vivo* on diabetic-induced endothelial dysfunction. *Life Sci.*, **60**, PL371-PL376.
- PIEPER, G.M. (1999). Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: importance of disease duration. *Diabetologia*, **42**, 204-213.
- PIERCE K.L., GIL, D.W., WOODWARD, D.F. & REGAN, J.W. (1995) cloning of human prostanoid receptors. *Trends Pharmacol. Sci.*, **16**, 253-256.
- PLANE, F. & GARLAND, C.J. (1996). Influence of contractile agonists on the mechanism of endothelium-dependent relaxation in rat isolated mesenteric artery. *Br. J. Pharmacol.*, **119**, 191-193.
- PLANE, F., HOLLAND, M., WALDRON, C.J., GARLAND, C.J. & BOYLE, J.P. (1997). Evidence that anandamide and EDHF act via different mechanisms in rat isolated mesenteric arteries. *Br. J. Pharmacol.*, **121**, 1509-1511.

- PLANE, F., PEARSON, T. & GARLAND, C.J. (1992). Differences between endothelium-dependent relaxation to acetylcholine and A23187 in the rabbit isolated femoral artery. *Br. J. Pharmacol.*, **107**, 201P.
- POHL, U. & BUSSES, R. (1989). Hypoxia stimulates release of endothelium-derived relaxant factor. *Am. J. Physiol.*, **256**, H1595-H1600.
- POPESCU, L.M., PANOIU, C., HINESOU, M., & NUTO, O. (1985). The mechanism of cGMP-induced relaxation in vascular smooth muscle. *Eur. J. Pharmacol.*, **107**, 393-394.
- PORTA, M., LA SELVA, M., MOLINATTI, P. & MOLINATTI, G.M. (1987). Endothelial cell function in diabetic microangiopathy. *Diabetologia*, **30**, 601-609.
- POSTON, L. & TAYLOR, P.D. (1995). Endothelium-mediated vascular function in insulin-dependent diabetes mellitus. *Clin. Sci.*, **88**, 245-255.
- PROPERZI, G., TERAGHI, G., GU, X-H., POCCIA, G., PASQUA, R., FRANCAVILLA, S. & POLAK, J.M. (1995). Early increase precedes a depletion of endothelin-1 but not of von Willebrand factor in cutaneous microvessels of diabetic patients. A quantitative immunohistochemical study. *J. Pathol.*, **175**, 243-252.
- QUIGNARD, J.-F., FELETOU, M., THOLON, C., VILANE, J.-P., DUHAULT, J. & VANHOUTTE, P.M. (1999). Potassium ions and endothelium-derived hyperpolarizing factor in guinea-pig carotid and porcine coronary arteries. *Br. J. Pharmacol.*, **127**, 27-34.
- RANDALL, M.D., ALEXANDER, S.P.H., BENNET, T., BOYD, E.A., FRY, J.R., GARDINER, S.M., KEMP, P.A., MCCULLOCH, A. & KENDAL, D.A. (1996).

- An exogenous cannabinoid as an endothelium-derived vasorelaxant. *Biochem. Biophys. Res. Commun.*, **229**, 114-120.
- RAPOPORT, R.M., DRAZNIN, M.B. & MURAD, F. (1983). Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature*, **306**, 174-176.
- REES, D.D., PALMER, R.M.J., HODSON, H.F. & MONCADA, S. (1989). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.*, **96**, 418-424.
- REES, D.D., PALMER, R.M.J., SCHULTZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746-752.
- REMBOLD, C.M. (1992). Regulation of contraction and relaxation in arterial smooth muscle. *Hypertension*, **20**, 129-137.
- ROSS, R., & GLOMSETT, J.A. (1976) The pathogenesis of atherosclerosis. *N.Engl. J. Med.*, **295** (7), 369-377 & 420-425.
- ROSS, R. (1993a) The pathogenesis of atherosclerosis. A perspective for the 1990s. *Nature*, **362**: 801-809.
- ROSS, R. (1993b) Rous-Whipple award lecture. Atherosclerosis: a defence mechanism gone awry. *Am. J. Pathol.*, **143** (4), 987-2002.
- ROSSINI, A.A., GREINER, D.L., FRIEDMAN, H.P. & MORDES, J.P. (1993). Immunopathogenesis of diabetes mellitus. *Diabetes Rev.*, **1**, 43-75.
- SAKAI, K., IMAIZUMI, T., MASAKI, H. & TAKESHITA, A. (1993). Intra-arterial infusion of insulin attenuates vasoreactivity in human forearm. *Hypertension*, **22**, 67-73.

- SANDEMAN, D.D., SHORE, A.C. & TOOKE, J.E. (1992). Relation of skin capillary pressure in patients with insulin-dependent diabetes mellitus to complications and metabolic control. *N. Eng. J. Med.*, **327**, 760-764.
- SHIBATA, S. (1969). Effect of prolonged cold storage on the contractile response of strips of rabbit aorta to various agents. *Circ. Res.*, **24**, 179-187.
- SHIMOKAWA, H., YASUTAKE, H., FUJII, K., OWADA, M.K., NAKAIKE, R., FUKUMOTO, Y., TAKAYANAGI, T., NAGAO, T., EGASHIRA, K., FUJISHIMA, M. & TAKESHITA, A. (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J. Cardiovasc. Pharmacol.*, **28**, 703-711.
- SIMPSON, H.C.R., STURLEY, R. STIRLING, C.A. & RECKLES, J.P.D. (1990). Combination of insulin with glipizide increases peripheral glucose disposal in secondary failure type 2 diabetic patients. *Diabetic Med.*, **7**, 143-147.
- SKIDGEL, R.A. & PRINTZ, M.P. (1978). PGI₂ production by rat blood vessels: diminished prostacyclin formation in veins compared to arteries. *Prostaglandins*, **16**, 1-16.
- SMITS, P., KAPMA, J.A., JACOBS, M.C., LUTTERMAN, J. & THIEN, T. (1993). Endothelium-dependent vascular relaxation in patients with type 1 diabetes. *Diabetes*, **42**, 148-153.
- SORENSEN, K.E., CELERMAJER, D.S., SPIEGELHALTER, D.J. ET AL (1995). Non-invasive measurement of endothelium-dependent arterial responses in man: accuracy and reproducibility. *Br. Heart J.*, **74**, 247-253.

- SQUADRITO, G.L. & PRYOR, W.A. (1995). The formation of peroxynitrite in vivo from nitric oxide and superoxide. *Chem. Biol. Interact.*, **96**, 203-206.
- STEVENS, M.J., FELDMAN, E.L. & GREENE, D.A. (1995). The aetiology of diabetic neuropathy: the combined roles of metabolic and vascular defects. *Diabetic Medicine*, **12**, 566-579.
- TADA, M. & KATZ, A.M. (1982). Phosphorylation of the sarcoplasmic reticulum and sarcolemma. *Annu. Rev. Physiol.*, **44**, 401-423.
- TAKAHASHI, K., GHATEI, M.A., LAM, H-C., O'HALLORAN, D.J. & BLOOM, S.R. (1990). Elevated plasma endothelin in patients with diabetes mellitus. *Diabetologia*, **33**, 306-310.
- TAKEDA, Y., MIYAMORI, I., YONEDA, T. & TAKEDA, R. (1991). Production of endothelin-1 from the mesenteric arteries of streptozotocin-induced diabetic rats. *Life Sci.*, **48**, 2553-2556.
- TATE, R.M., MORRIS, H.G., SCHROEDER, W.R. & REPINE, J.E. (1984). Oxygen metabolites stimulate thromboxane production and vasoconstriction in isolated saline-perfused rabbit lungs. *J. Clin. Invest.*, **74**, 608-613.
- TATESON, J.E., MONCADA, S. & VANE, J.R. (1977) Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. *Prostaglandins*, **13**, 389-397.
- TAYLOR, P.D. & POSTON, L. (1994). The effect of hyperglycaemia on function of rat isolated mesenteric resistance artery. *Br. J. Pharmacol.*, **113**, 801-808.
- TAYLOR, P.D., GRAVES, J.E. & POSTON, L. (1995). Selective impairment of acetylcholine-mediated endothelium-dependent relaxation in isolated resistance arteries of the streptozotocin-induced diabetic rats. *Clin. Sci.*, **88**, 519-524.

- TAYLOR, P.D., MCCARTHY, A.L., THOMAS, C.R. & POSTON, L. (1992). Endothelium-dependent relaxation and noradrenaline sensitivity in mesenteric resistance arteries of streptozotocin-induced diabetic rats. *Br. J. Pharmacol.*, **107**, 393-399.
- TAYLOR, P.D., OON, B., THOMAS, C.R. & POSTON, L. (1994). Prevention by insulin treatment of endothelial dysfunction but not enhanced noradrenaline-induced contractility in mesenteric resistance arteries from streptozotocin-induced diabetic rats. *Br. J. Pharmacol.*, **111**, 35-41.
- TAYLOR, S.G. & WESTON, A.H. (1988). Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol. Sci.*, **9**, 272-274.
- TESFAMARIAM, B. & COHEN, R.A. (1992). Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am. J. Physiol.*, **263** (Heart Circ. Physiol., 32), H321-H326.
- TESFAMARIAM, B. (1994). Free radicals in diabetic endothelial cell dysfunction. *Free Radic. Biol. Med.*, **16**, 383-391.
- TESFAMARIAM, B., BROWN, M.L. & COHEN, R.A. (1991). Elevated glucose impairs endothelium-dependent relaxation by activating protein kinase C. *J. Clin. Invest.*, **87**, 1643-1648.
- TESFAMARIAM, B., BROWN, M.L., DEYKIN, D. & COHEN, R.A. (1990). Elevated glucose promotes generation of endothelium-derived vasoconstrictor prostanoids in rabbit aorta. *J. Clin. Invest.*, **85**, 929-932.

- TESFAMARIAM, B., JAKUBOWSKI, J.A. & COHEN, R.A. (1989). Contraction of diabetic rabbit aorta caused by endothelium-derived PGH₂-TxA₂. *Am. J. Physiol.*, **257** (Heart Circ. Physiol., 26), H1327-H1333.
- TESFAMARIAM, B., PALACINO, J.J., WEISBROD, R.M. & COHEN, R.A. (1993). Aldose reductase inhibition restores endothelial cell function in diabetic rabbit aorta. *J. Cardiovasc. Pharmacol.*, **21**, 205-211.
- TOMLINSON, K.C., GARDINER, S.M., HEBDEN, R.A. & BENNETT, T. (1992). Functional consequences of streptozotocin-induced diabetes mellitus, with particular reference to the cardiovascular system. *Pharmacol. Rev.*, **44**, 103-150.
- TOOKE, J.E. (1995) Microvascular function in human diabetes. A physiological perspective. *Diabetes*, **44**, 721-726.
- TOOKE, J.E. (1996). Microvasculature in diabetes. *Cardiovasc. Res.*, **32**, 764-771.
- TOROK, J., KRISTEK, F. & MOKRASOVA, M. (1993). Endothelium-dependent relaxation in rabbit aorta after cold storage. *Eur. J. Pharmacol.*, **228**, 313-319.
- TRUGLIA, J.A., LIVINGSTON, J.N. & LOCKWOOD, D.H. (1985). Insulin resistance: receptor and post-binding defects in human obesity and non-insulin-dependent diabetes mellitus. *Am. J. Med.*, **79** (Suppl B), 13-22.
- TUCKER, B.J. (1990). Early onset of increased transcapillary albumin escape in awake diabetic rats. *Diabetes*, **39**, 919-923.
- URAKAMI-HARASAWA, L., SHIMOKAWA, H., NAKASHIMA, M., EGASHIRA, K. & TAKESHITA, A. (1997). Importance of endothelium-derived hyperpolarizing factor in human arteries. *J. Clin. Invest.*, **100**, 2793-2799.
- VALLANCE, P., COLLIER, J. & MONCADA, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet*, **2**, 997-1000.

- VANHOUTTE, P.M. (1990) Endothelium-derived relaxing and contracting factors: pathological and therapeutic implications. *Eur. J. Pharmacol.*, **183** (1), 109-110.
- VANHOUTTE, P.M. & MILLER, V.M. (1985) Heterogeneity of endothelium-dependent responses in mammalian blood vessels. *J. Cardiovasc. Pharmacol.*, **7** (Suppl. 3), S12-S23.
- VANHOUTTE, P.M. (1989). Endothelium and the control of vascular function. *Hypertension*, **13**, 658-667.
- VOHRA, M.M., CHEDRAWY, E.G., LI, G. & SULLIVAN, J.A. (1997). Functional assessment of rabbit thoracic aorta stored in University of Wisconsin, St. Thomas' Hospital and Krebs solutions. *Br. J. Pharmacol.*, **120**, Proc. Suppl.,153P.
- WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.*, **39**, 163-196.
- WALKER, A.B., SAVAGE, M.W., DORES, J. & WILLIAMS, G. (1997). Insulin-induced attenuation of noradrenaline-mediated vasoconstriction in resistance arteries from Wistar rats is nitric oxide dependent. *Clin. Sci.*, **92**, 147-152.
- WALLERSTEDT, S.M. & BODELSSON, M. (1997). Endothelium-dependent relaxation by substance P in human isolated omental arteries and veins: relative contribution of prostanoids, nitric oxide and hyperpolarization. *Br. J. Pharmacol.*, **120**, 25-30.
- WALSH, M.P. (1993). Regulation of vascular smooth muscle tone. *Can. J. Physiol. Pharmacol.*, **72**, 919-936.
- WAMBACH, G.K. & LIU, D. (1991). Insulin attenuates vasoconstriction induced by noradrenaline, serotonin and potassium chloride in rat mesenteric resistance arteries. *J. Hypertens.*, **9** (suppl 6), S198-S199.

- WANNER, T.D., MITCHELL, J.A., DENUCCI, G. & VANE, J.R. (1989). Endothelin-1 and endothelin-3 release EDRF from isolated perfused arterial vessels of the rat and rabbit. *J. Cardiovasc. Pharmacol.*, **13** (suppl 5), S85-S88.
- WATTS, G.F., O'BRIEN, S.F., SILVESTER, W. & MILLAR, J.A. (1996). Impaired endothelium-dependent and independent dilatation of forearm resistance arteries in men with diet-treated non-insulin-dependent diabetes: role of dyslipidaemia. *Clin. Sci.*, **91**, 567-573.
- WENNMALM, A., BENTHIN, G., EDLUND, A. ET AL (1993). Metabolism and excretion of nitric oxide in humans. An experimental and clinical study. *Circ. Res.*, **73**, 1121-1127.
- WHITE, R.E. & CARRIER, G.O. (1990). Vascular contraction induced by activation of membrane calcium ion channels in streptozotocin diabetes. *J. Pharmacol. Exp. Ther.*, **253**, 1057-1062.
- WILLIAMS, S.B., GOLDFINE, A.B., TIMIMI, F.K., TING, H.H., RODDY, M-A., SIMONSON, D.C. & CREAGER, M.A. (1998). Acute hyperglycaemia attenuates endothelium-dependent vasodilation in humans *in vivo*. *Circulation*, **97**, 1695-1701.
- WILLIAMSON, J.R., OSTROW, E., EADES, D., CHANG, K., ALLISON, W., KILO, C. & SHERMAN, W.R. (1990). Glucose-induced microvascular functional changes in nondiabetic rats are stereospecific and are prevented by an aldose reductase inhibitor. *J. Clin. Invest.*, **85**, 1167-1172.
- WOLF, B.A., WILLIAMSON, J.R., EASOM, R.A., CHANG, K., SHERMAN, W.R. & TURK, J. (1991). Diacylglycerol accumulation and microvascular abnormalities induced by elevated glucose levels. *J. Clin. Invest.*, **87**, 31-38.

- WOLFF, S.P. (1993). Diabetes mellitus and free radicals. Free radicals, transition metals and oxidative stress in the aetiology of diabetes mellitus and complications. *Br. Med. Bull.*, **49**, 642-652.
- WOOLFSON, R.G. & POSTON, L. (1990). Effect of N^G-monomethyl-L-arginine on endothelium-dependent relaxation of human subcutaneous resistance arteries. *Clin. Sci.*, **79**, 273-278.
- WORLD HEALTH ORGANISATION (WHO) STUDY GROUP (1985). Diabetes Mellitus (Technical Report Series 727). World Health Organization, Geneva.
- WORLD HEALTH ORGANISATION, DEPARTMENT OF NONCOMMUNICABLE DISEASE SURVEILLANCE (1999). Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications. World Health Organization, Geneva
- WU, H.Y., JENG, Y.Y., YUE, C.J., CHYU, K.Y., HSEUH, W.A. & CHAN, T.M. (1994). Endothelial-dependent vascular effects of insulin and insulin-like growth factor 1 in the perfused rat mesenteric artery and aortic rings. *Diabetes*, **43**, 1027-1032.
- WU, M., PRITCHARD JR, K.A. & KAMINSKI, P.M. (1994). Involvement of nitric oxide and nitrosothiols in relaxation of pulmonary arteries to peroxynitrite. *Am. J. Physiol.*, **266**, H2108-H2113.
- YAGI, S., TAKATA, S., KIYOKAWA, H., YAMAMOTO, M., NOTO, Y., IKEDA, T. & HATTORI, N. (1988). Effects of insulin on vasoconstrictive responses to norepinephrine and angiotensin II in rabbit femoral artery and vein. *Diabetes*, **37**, 1064-1067.

- YAMAUCHI, T., OHNAKA, K., TAKATANAGI, R., UMEDA, F. & NAWATA, H. (1990). Enhanced secretion of endothelin-1 by elevated glucose levels from cultured bovine aortic endothelial cells. *FEBS Lett.*, **267**, 16-18.
- YANAGISAWA, M., KIRIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature (Lond)*, **332**, 411-415.
- YASUDA, H., TANIGUCHI, Y., YAMASHITA, M., KOBAYASHI, N., SONOBE, M., HATANAKA, I., KIKKAWA, R. & SHIGETA, Y. (1990). Morphological characteristics of dermal diabetic microangiopathy. *Diabetes Res. Clin. Pract.*, **9**, 187-194.
- ZENERE, B.M., ARCARO, G., SAGGIANI, F., ROSSI, L., MUGGEO, M. & LEHI, A. (1995). Non-invasive detection of functional alterations of the arterial wall in IDDM patients with and without microalbuminuria. *Diabetes Care*, **18**, 975-982.
- ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1996). Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. *Br. J. Pharmacol.*, **117**, 1600-1606.
- ZYGMUNT, P.M., WALDECK, K. & HÖGESTÄTT, E.D. (1994). The endothelium mediates a nitric oxide-independent hyperpolarization and relaxation in the rat hepatic artery. *Acta. Physiol. Scand.*, **152**, 375-384.



ELSEVIER
SCIENCE

5 July 2002

Our ref: HW/jj/Jul02/045

Your ref:

Carol-Ann McIntyre
C/o Patrick W F Hadoke
Endocrinology Unit
Department of Medical Sciences
2nd Floor OPD Building
Western General Hospital
Edinburgh E114 2XU
Scotland

Dear Carol-Ann McIntyre

**GENERAL PHARMACOLOGY, (now VASCULAR PHARMACOLOGY), Vol 35, No 3, 2001, pp 119-127,
Hadoke et al: "Functional heterogeneity of large and small resistance ..."**

As per your letter dated 18 June 2002, we hereby grant you permission to reprint the aforementioned material at no charge in your thesis subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.
2. Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier Science".
3. Reproduction of this material is confined to the purpose for which permission is hereby given.
4. This permission is granted for non-exclusive world **English** rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form. Should you have a specific electronic project in mind please reapply for permission.
5. Should your thesis be published commercially, please reapply for permission.

Yours sincerely


Helen Wilson
Rights Manager

**Your future requests will be handled more quickly if you complete the online form at
www.elsevier.com**

Elsevier Science, Global Rights Department, PO Box 800, Oxford OX5 1DX, UK
Tel +44 (0)1865 843830 | Fax +44 (0)1865 853333 | www.elsevier.com
vat no. 494 6272 12

Academic Press • Butterworth-Heinemann • Cell Press • Churchill Livingstone • Engineering Information • Excerpta Medica
The Lancet • MDConsult • MDL • Mosby • North-Holland • Pergamon • ScienceDirect • WB Saunders

Functional heterogeneity of large and small resistance arteries isolated from biopsies of subcutaneous fat

Implications for investigation of vascular pathophysiology

Patrick W.F. Hadoke^{a,*}, Carol-Ann McIntyre^{a,b}, Gillian A. Gray^c, Christine H. Buckley^b

^aDepartment of Medical Sciences, Western General Hospital, Hugh Robson Building, University of Edinburgh, Crewe Road, Edinburgh EH4 2XU, Scotland, UK

^bDepartment of Medicine, Royal Infirmary, Hugh Robson Building, University of Edinburgh, Edinburgh, UK

^cDepartment of Biomedical Sciences, Hugh Robson Building, University of Edinburgh, Edinburgh, UK

Received 1 October 2000; received in revised form 1 April 2001; accepted 1 June 2001

Abstract

Few studies using human subcutaneous resistance arteries acknowledge the possibility of functional heterogeneity in these vessels. Large ($\sim 500 \mu\text{m}$) and small ($\geq 200 \mu\text{m}$) resistance arteries ($n=11$) and veins ($n=5$) were identified using physical, structural and functional criteria in 14 biopsies of human gluteal fat. Endothelium-dependent relaxation was not evident in veins, while, unlike small resistance arteries ($E_{\text{max}} 95.74 \pm 1.86\%$; $-\log \text{IC}_{50} 7.28 \pm 0.09$), large resistance arteries with an intact endothelium failed to respond to acetylcholine. These results suggest that large resistance arteries may lack muscarinic receptors on the endothelium and emphasise the importance of careful vessel selection and characterisation in studies using human resistance arteries. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Endothelium; Human resistance arteries; Acetylcholine; Vascular reactivity

1. Introduction

Isolated vessel studies have consistently shown that the development of cardiovascular disease is associated with impaired function of the endothelium (Vanhoutte, 1989; Chan et al., 2000). Many of these investigations have used vessels isolated from experimental animals, but since the introduction of the small vessel myograph, an increasing number of studies have been performed using arteries from human subcutaneous fat. This approach has the advantage of studying vascular reactivity directly in vessels from patients and uses resistance arteries, which contribute significantly to maintenance of blood pressure (Mulvany and Aalkjaer, 1990). Human subcutaneous resistance arteries have now been used to investigate vascular dysfunction in a variety of conditions, including hypertension (Angus et al., 1992), diabetes (McNally et al., 1994; Cipolla et al., 1996; McIntyre et al., 1998a,b), glaucoma (Buckley et al., 1999), pre-

eclampsia (Aalkjaer et al., 1985), congestive heart failure (Angus et al., 1993), hypercholesterolaemia (Lewis et al., 1998) and Raynaud's disease (Smith et al., 1999).

In many studies, endothelium-dependent relaxation of human gluteal resistance arteries is assessed using acetylcholine (ACh). This method has limitations, however, as an early investigation demonstrated that, unlike small resistance arteries (internal diameter $\leq 200 \mu\text{m}$), large resistance arteries (internal diameter $\sim 500 \mu\text{m}$) from this source fail to respond to ACh despite having an intact endothelium (Angus et al., 1992). The mechanism responsible for this functional variation was not identified, but could result from heterogeneity in receptor populations and/or endothelium-derived relaxing factor activity (Vanhoutte and Miller, 1985; Shimokawa et al., 1996; Ohkubo and Chiba, 1988). This variation in ACh-mediated relaxation in human gluteal resistance arteries has significant implications for studies of disease pathogenesis, particularly those which identify a selective impairment in the responsiveness to ACh (McNally et al., 1994; Lewis et al., 1998). Surprisingly, however, many studies do not address the possibility that ACh-insensitive resistance arteries may have been included in functional investigations. This may be due to a lack of

* Corresponding author. Tel.: +44-131-537-1742; fax: +44-131-537-1012.

E-mail address: phadoke@srv0.med.ed.ac.uk (P.W.F. Hadoke).

awareness as functional heterogeneity in human subcutaneous resistance arteries has not been investigated in detail and is referred to only indirectly in a study of hypertension (Angus et al., 1992). Consequently, the aims of this investigation were (1) to identify the mechanisms responsible for the inability of large resistance arteries to relax following stimulation with ACh, and (2) to describe a simple procedure to aid selection of the appropriate artery from the different vessels isolated from human gluteal fat.

2. Materials and methods

This investigation made use of surplus vessels isolated from biopsies taken for the study of vascular function in insulin-dependent diabetes mellitus (IDDM), noninsulin-dependent diabetes mellitus (NIDDM) or normal pressure glaucoma (NPG). Biopsies were taken from 14 individuals (age 58 ± 2 years; range 43–69 years; sex 12 males, 2 females) who were either control subjects ($n=5$) or were suffering from NIDDM ($n=5$), IDDM ($n=2$) or NPG ($n=2$). We have previously shown that functional responses of resistance arteries to noradrenaline (NA), ACh, bradykinin (BK) and the nitric oxide donor 3'-morpholiniosydnonimine (SIN-1) are not altered in patients with these conditions (McIntyre et al., 1998a,b; Buckley et al., 1999). All functional experiments were performed on the day following biopsy, and preliminary studies (data not shown) confirmed that responses to ACh and BK, but not those to SIN-1, were abolished following removal of the endothelium. The patients and controls enrolled for these studies all provided informed, written consent and the procedure was approved by the Lothian Research Ethics Committee.

A biopsy of skin and subcutaneous fat [approximately 2 (L) \times 1 (W) \times 1 (H) cm] was taken under local anaesthesia from the gluteal region of each individual (5 ml of 2% lignocaine hydrochloride; Astra, Hertfordshire, UK). Vessels were dissected from these biopsies using light microscopy and were identified as either small resistance arteries, large resistance arteries or veins by their distinctive physical characteristics: small resistance arteries have a smooth adventitial surface, maintain their shape when removed from physiological salt solution (PSS) and the vessel wall is sufficiently transparent to allow distinction between the media and lumen (Fig. 1a(i)); this contrasts with veins which have a "furry" adventitial margin, do not maintain their shape (i.e., they "roll up") when removed from PSS and in which the margin between media and lumen cannot be distinguished through the vessel wall (Fig. 1b(i)); large resistance arteries are identified by their greater diameter, compared with small resistance arteries. They maintain their shape when removed from PSS, but, unlike small resistance arteries, have a "furry" adventitial surface and the wall is not sufficiently transparent to allow distinction between media and lumen (Fig. 1c(i)). Mor-

phological and functional studies were also performed on veins to allow direct comparison with large and small arteries. Although large and small diameter veins were isolated, functional and morphological studies were performed using only those which were similar in size to the small resistance arteries (Fig. 1).

2.1. Drugs

All salts were obtained from BDH (Poole, Dorset, UK). NA hydrochloride, ACh chloride, calcium ionophore A23187 and BK acetate were obtained from Sigma (Poole, Dorset, UK). SIN-1 and endothelin-1 (ET-1) were obtained from Alexis (Nottingham, UK). Stock solutions (10^{-3} M) were prepared in distilled water, except for ET-1 and A23187, which were initially dissolved in 50% methanol and absolute ethanol, respectively, to give 10^{-5} and 10^{-4} M stock solutions. Subsequent dilutions were made in distilled water and final bath concentrations of methanol and ethanol did not exceed 1.5% and 3% v/v, respectively. Stock solutions of all drugs were frozen at -20°C as 1 ml aliquots and thawed as required.

2.2. Morphological investigations

Fifteen small and seven large resistance arteries, as well as four veins (from 10, 7 and 4 individuals, respectively), were fixed in 10% neutral buffered formalin, dehydrated in ethanol and chloroform and embedded in paraffin for subsequent histological analysis. This procedure was performed using both freshly isolated vessels and some which had been used for functional investigation. Serial 3 μm transverse sections were stained using haematoxylin and eosin and immunocytochemical techniques. Smooth muscle and endothelial cells were detected using mouse anti α -smooth muscle actin IgG (1/50 dilution; Novocastra Laboratories, Newcastle upon Tyne, UK) and biotinylated ulex europaeus agglutinin (1/100 dilution; Vector Laboratories, Peterborough, UK), respectively. α -SMA IgG (but not ulex, which was already biotinylated) was incubated with a biotinylated secondary antibody (Dako Ltd., Ely, UK). Both smooth muscle and endothelium were detected using a streptavidin-biotin-peroxidase kit (Dako Ltd., Ely, UK) followed by incubation with diaminobenzidine. Positive signals appeared brown. Negative controls were obtained by omitting the primary antibody.

2.3. Functional investigation

Eleven biopsies (nine males, two females; age 58 ± 9 years; four control, three NIDDM, two IDDM, two NPG) yielded both large and small resistance arteries, while veins were isolated from five male subjects (age 54 ± 4 years; three NIDDM, one IDDM, one control). Isolated vessels were mounted as ring preparations on two 40- μm stainless steel wires in a small vessel myograph (J.P. Trading, Aarhus,

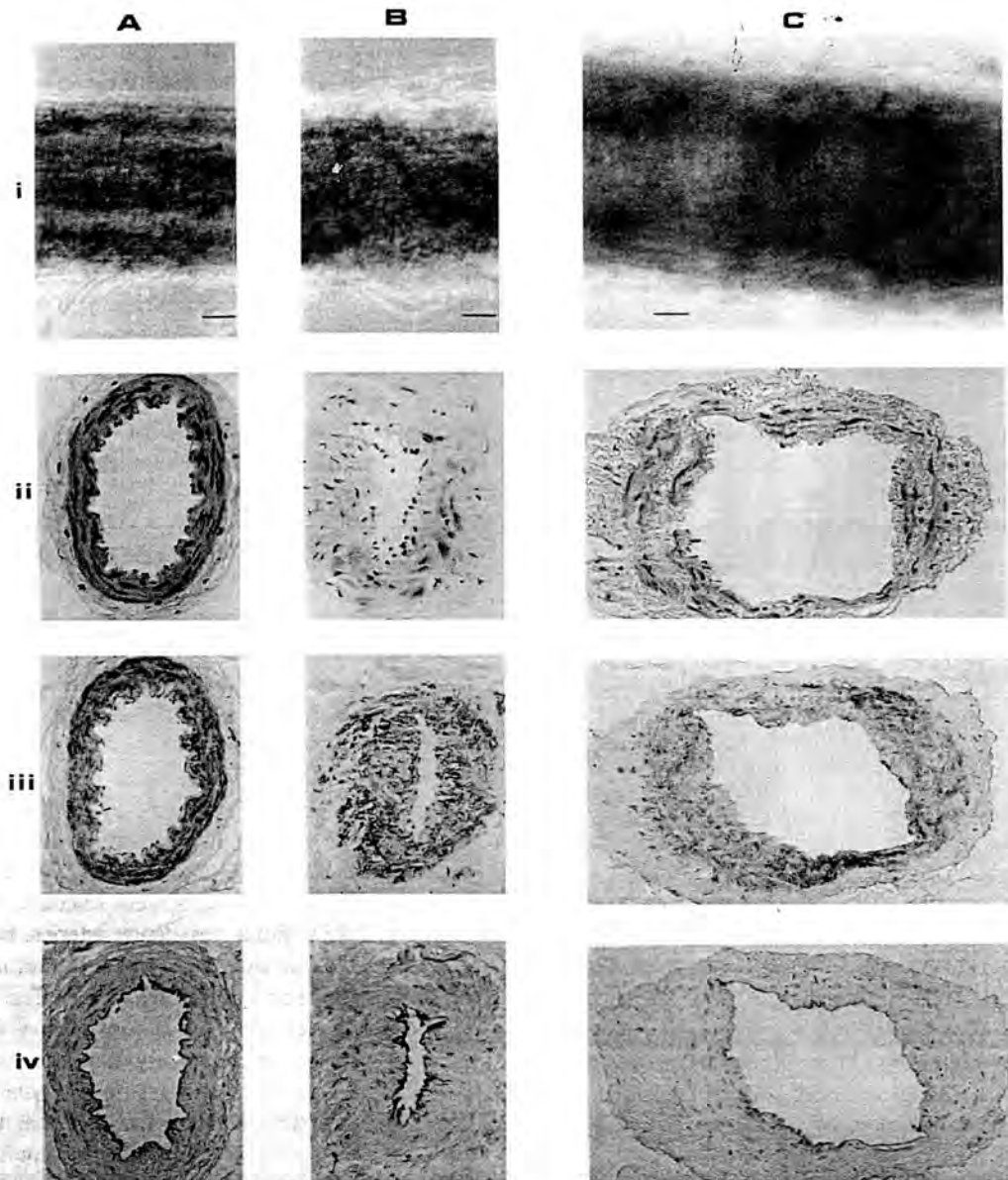


Fig. 1. Physical and morphological differences between small resistance arteries (A), veins (B) and large resistance arteries (C) isolated from biopsies of human subcutaneous fat. Vessels were (i) photographed under light microscopy (scale bar = 50 μ m), while photomicrographs were taken following staining with (ii) haematoxylin and eosin, and with antibodies against (iii) α -smooth muscle actin (iv) and ulex (for detection of the endothelium). Magnification $\times 200$ for all photomicrographs. The large resistance artery was fixed following functional studies in the wire myograph. This accounts for the rather distorted appearance of this vessel and may also contribute to the relatively weak staining for α -smooth muscle actin and endothelium. This diagram is representative of results obtained using 15 small and 7 large resistance arteries and 4 veins obtained from 10, 7 and 4 individuals, respectively.

Denmark). One wire was fixed to a movable micrometer, while the other was attached to an isometric force transducer. The vessels were immersed in PSS (composition (mM): NaCl 119, KCl 4.7, CaCl_2 2.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.17, KH_2PO_4 1.18, NaHCO_3 25, K_2EDTA 0.026 and D-glucose 5.5) at 37°C and perfused continuously with 95% O_2 and 5% CO_2 . The length of each vessel segment was measured using a travelling micrometer eyepiece attached to a light microscope. Following an equilibration period (30 min), the vessels were subjected to a standard normalisation procedure (Mulvany and Halpern, 1977) in which each is exposed

to incremental stretches. Application of the Laplace equation calculates the internal circumference (L_{100}) the vessel would have in vivo when relaxed and subjected to a pressure of 13.3 kPa (100 mm Hg). The vessels were then stretched to the optimum resting setting used for human resistance arteries ($0.9L_{100}$; Angus et al., 1992; McNally et al., 1994).

Vascular rings were equilibrated at their optimum resting setting for 30 min and were then subjected to a standard start procedure. This consisted of five consecutive applications of vasoconstrictors. After each expo-

sure, the artery was washed several times with PSS and the responses were allowed to return to baseline. The first, second and fifth contractions were produced using NA-K [a 125 mM potassium solution (KPSS, made by equimolar substitution of KCl for NaCl in PSS) containing NA (10^{-5} M)]. The third contraction was producing using KPSS, while the fourth was obtained using NA (10^{-5} M). This procedure confirmed the viability of each artery and demonstrated the reproducibility of the vasoconstrictor responses.

Cumulative concentration–response curves were obtained in all three vessel types using the receptor-dependent vasoconstrictors NA (10^{-9} – 3×10^{-5} M) and ET-1 (10^{-11} – 3×10^{-7} M) and the receptor-independent vasoconstrictor K^+ (10–125 mM). Cumulative concentration–response curves were obtained for the receptor-dependent, endothelium-dependent relaxant agonists ACh (10^{-9} – 3×10^{-5} M) and BK (10^{-10} – 3×10^{-6} M), the receptor-independent, endothelium-dependent dilator, A23187 (10^{-9} – 3×10^{-6} M) and the endothelium-independent nitric oxide donor, SIN-1 (10^{-9} – 3×10^{-5} M). Responses to vasodilators were obtained following contraction of the artery with sufficient NA (3×10^{-7} – 3×10^{-6} M) to produce 50–80% of the maximal response to this agonist.

2.4. Statistics

All values are mean \pm S.E.M. for n experiments, where n represents the number of subjects. Contractile responses are expressed as milliNewtons per millimeter and relaxation as a percentage of the pre-contraction with NA. The concentration of agonist required to produce 50% of the maximum response (EC_{50}) was obtained by fitting the Hill equation to the data using curve fitting software (Fig P; Biosoft, Cambridge, UK). The results are expressed as the negative logarithm of the EC_{50} (pD_2 for vasoconstrictors, $-\log IC_{50}$ for vasodilators). Maximum response and EC_{50} values were compared using one-way analysis of variance followed by a Tukey's post-hoc test and significance was assumed when $P < .05$.

3. Results

Large and small resistance arteries, and small veins isolated from the biopsies of subcutaneous fat were distinguished using physical differences as described in the Materials and Methods section.

3.1. Morphology

Small ($\leq 200 \mu\text{m}$) resistance arteries (Fig. 1a) had a clearly defined media and adventitia when stained with haematoxylin and eosin (Fig. 1a(ii)). Immunohistochemistry demonstrated that the media comprised mainly smooth

muscle cells (Fig. 1a(iii)) with a monolayer of endothelial cells lining the internal elastic lamina (Fig. 1a(iv)). In contrast, veins failed to hold their shape, and distinction between media and adventitia was less clear when stained with haematoxylin and eosin (Fig. 1b(ii)). However, the venous media stained strongly for α -SMA (Fig. 1b(iii)) and the ulex antibody identified a clear endothelium lining the lumen (Fig. 1b(iv)). The large ($\sim 500 \mu\text{m}$) resistance arteries had more structural similarity with the small resistance arteries than the veins as they held their shape and had clearly defined medial and adventitial layers following staining with haematoxylin and eosin (Fig. 1c(ii)). As with both the small resistance artery and the vein, the media was comprised mainly of smooth muscle cells (Fig. 1c(iii)) and an endothelial cell layer was detected on the luminal surface (Fig. 1c(iv)).

3.2. Normalisation

Normalisation of small resistance arteries was straightforward with the effective transmural pressure exceeding 13.3 kPa after three to four stretches. In contrast, large resistance arteries and veins did not maintain stretch as well as small resistance arteries and required more (6–10) equivalent stretches. The internal diameter of large resistance arteries ($485 \pm 56 \mu\text{m}$; $n = 11$) was bigger ($P = .00005$) than that of the small resistance arteries ($185 \pm 16 \mu\text{m}$) when stretched to an effective pressure of 13.3 kPa (100 mm Hg). Interestingly, although veins were selected, which appeared similar in size to small resistance arteries, they were found to have an internal diameter ($410 \pm 93 \mu\text{m}$; $n = 5$) similar to the large resistance arteries when an effective pressure of 13.3 kPa was exerted. This is consistent with the tendency of veins to distend more readily than arteries in response to small increases in internal pressure (Bell and Emslie-Smith, 1980) and probably reflects the fact that the veins were being studied under nonphysiological conditions to allow direct comparison with arteries.

3.3. Vascular function

NA, ET-1 and KPSS produced concentration-dependent contractions in all three types of vessel (Fig. 2). There was a trend towards a greater response to all three vasoconstrictors in the large resistance arteries, but in most cases, this did not reach significance. Furthermore, this apparent difference was lost when responses were corrected to take into account differences in internal diameter (Table 1). The response to NA (Fig. 2a) in the large resistance arteries ($E_{\max} 3.11 \pm 0.52$ mN/mm; $n = 11$) was not significantly bigger than that produced in small resistance arteries (2.02 ± 0.36 mN/mm; $n = 10$; $P = .17$) or veins (1.65 ± 0.24 mN/mm; $n = 5$; $P = .14$). The sensitivity (pD_2) of large resistance arteries to NA (7.40 ± 0.14) was identical to that exhibited by veins (7.40 ± 0.14 ; $P = 1.00$) and not significantly different from that of small (7.01 ± 0.17 ; $P = .18$) resistance arteries.

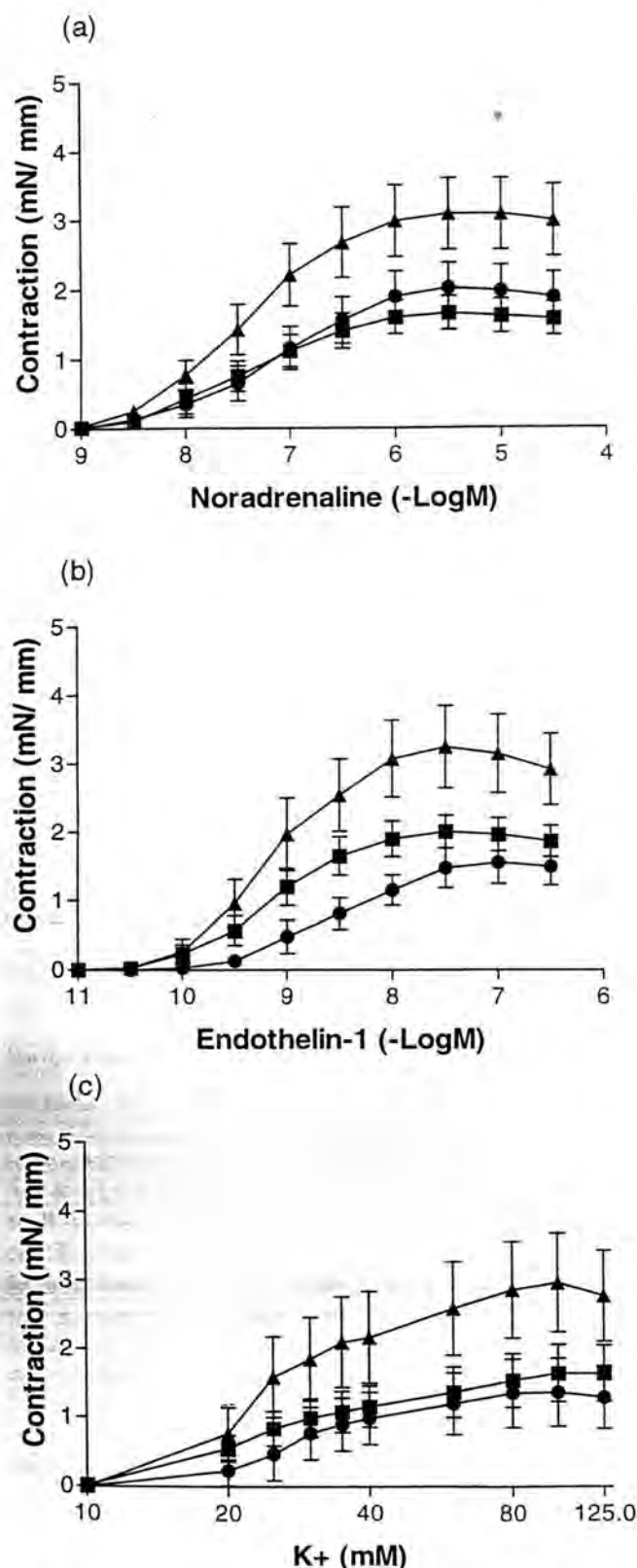


Fig. 2. Concentration–response curves to (a) NA, (b) endothelin-1 and (c) K^+ in small resistance arteries (\bullet , $n=10$), veins (\blacksquare , $n=5$) and large resistance arteries (\blacktriangle , $n=11$) isolated from human subcutaneous fat biopsies. Each point represents mean \pm S.E.M.

The contraction (E_{\max}) produced by ET-1 (Fig. 2b) was stronger ($P=.02$) in large (3.35 ± 0.60 mN/mm; $n=7$) compared with small (1.57 ± 0.30 mN/mm; $n=7$) resistance arteries, but not significantly different ($P=.13$) than the response produced in veins (2.00 ± 0.24 mN/mm; $n=5$). The difference between large and small resistance arteries was lost when the results were expressed as a percentage of the maximum response to NA-K (Table 1). The trend towards greater sensitivity in large resistance arteries (pD_2 9.05 ± 0.16) and veins (9.19 ± 0.20) compared with small resistance arteries (8.61 ± 0.17) was not significant ($P=.19$ and $P=.85$, respectively).

As with NA and ET-1, the apparently greater E_{\max} to KPSS (Fig. 2c) produced by large resistance arteries (2.99 ± 0.72 mN/mm; $n=7$) was not significant when compared with small resistance arteries (1.39 ± 0.49 mN/mm; $n=7$; $P=.14$) and veins (1.67 ± 0.41 mN/mm; $n=7$; $P=.31$). Furthermore, the sensitivity (pD_2) to this constrictor in large resistance arteries (1.53 ± 0.04) was not different to that in small resistance arteries (1.51 ± 0.04 ; $P=.90$) and veins (1.58 ± 0.06 ; $P=.71$).

BK-mediated relaxation (Fig. 3a) was similar in small (E_{\max} $88.35 \pm 4.76\%$; $-\log IC_{50}$ 7.49 ± 0.15 ; $n=9$) and large (E_{\max} $78.67 \pm 3.92\%$; $P=.26$; $-\log IC_{50}$ 7.88 ± 0.17 ; $P=.11$; $n=11$) resistance arteries. In contrast, relaxation of veins in response to BK (E_{\max} $21.68 \pm 5.60\%$; $n=5$) was significantly smaller than responses produced in the small ($P=.0001$) and large ($P=.0001$) resistance arteries, although the sensitivity of this response was similar ($-\log \log IC_{50}$ 8.20 ± 0.30 ; $n=4$; $P=.09$ and $.57$, respectively). Exposure to ACh (Fig. 3b) did not produce an active relaxation in large resistance arteries or veins despite demonstrating its ability to relax small resistance arteries (E_{\max} $95.74 \pm 1.86\%$; $-\log IC_{50}$ 7.28 ± 0.09 ; $n=11$). Conversely, A23187 (Fig. 3c) caused similar relaxation responses in large (E_{\max} $67.00 \pm 8.37\%$; $-\log IC_{50}$ 6.63 ± 0.20) and small (E_{\max} $76.00 \pm 11.52\%$; $P=.84$; $-\log IC_{50}$ 6.76 ± 0.23 ; $P=.66$; $n=6$) resistance arteries but only evoked relaxation in two out of five veins. The relaxation responses in the two veins, which responded to A23187 (E_{\max} 52.88% and 91.18% ; $-\log IC_{50}$ 6.82 and 6.37), were similar to those produced in large and small arteries by this ionophore.

Table 1

Maximum contractile responses (E_{\max}) of small and large resistance arteries and veins to NA and ET-1

	E_{\max} (% NA-K)	
	NA	ET-1
Small resistance artery	102.84 ± 5.42 (10)	92.98 ± 10.96 (7)
Vein	96.12 ± 3.41 (5)	124.19 ± 35.56 (5)
Large resistance artery	104.61 ± 5.11 (11)	111.90 ± 21.23 (7)

Data are mean \pm S.E.M. of (n) observations and are expressed as a percentage of the maximum response produced by NA-K (a solution containing 125 mM K^+ and 10^{-5} M NA) to control for variations in vessel size.

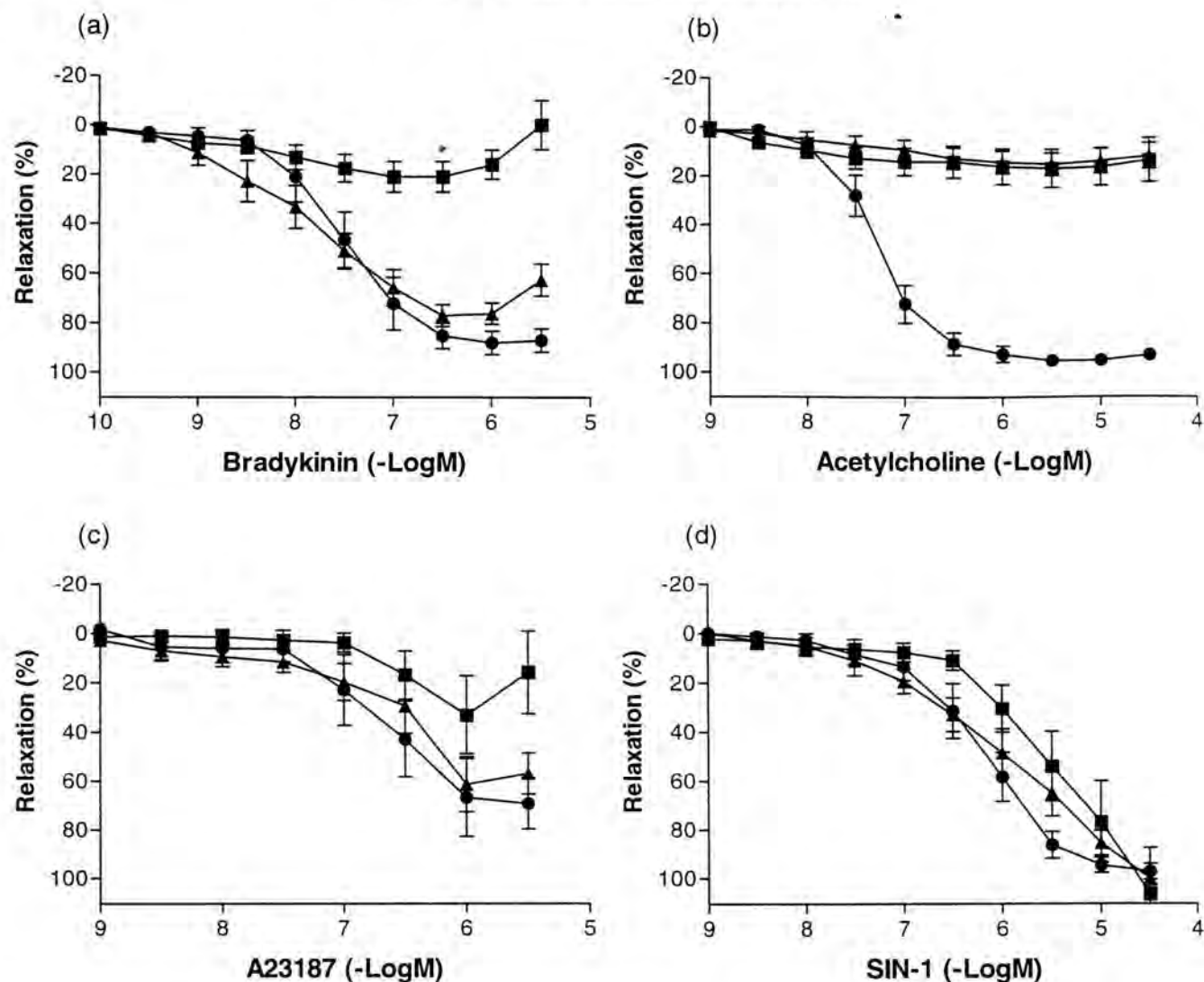


Fig. 3. Concentration–response curves to the vasodilators (a) BK, (b) ACh (c) A23187 and (d) SIN-1 in resistance arteries (●, $n=9-10$), veins (■, $n=5$) and large resistance arteries (▲, $n=7-11$) isolated from human subcutaneous fat biopsies. Each point represents mean \pm S.E.M.

All three vessel types relaxed in response to SIN-1 (Fig. 3d). The magnitude (E_{\max} 100.15 ± 2.75 ; $n=10$) and sensitivity ($-\log IC_{50}$ 5.98 ± 0.18) of this response in large resistance arteries were similar to those produced by small resistance arteries ($98.70 \pm 2.64\%$; $n=9$; $P=.99$; and 6.19 ± 0.14 ; $P=.55$; respectively). However, while SIN-1 produced a response of similar magnitude ($109.00 \pm 18.52\%$; $n=5$; $P=.69$) in veins, the sensitivity of this response ($-\log IC_{50}$ 5.52 ± 0.11) was significantly lower ($P=.04$) than in small, but not large ($P=.23$), resistance arteries.

4. Discussion

This investigation confirmed functional heterogeneity between large and small resistance arteries obtained from biopsies of human, subcutaneous, gluteal fat, with large resistance arteries unable to relax in response to the

endothelium-dependent vasorelaxant, ACh. Functional analyses suggested that this was the result of the endothelial cells of large resistance arteries, unlike those in their smaller counterparts, lacking muscarinic receptors. These results highlight the importance of correct identification of resistance arteries isolated from gluteal fat biopsies. Comparison of small veins and large and small resistance arteries obtained from this source has provided both physical and functional criteria for ensuring selection of the required vessel type.

Vasorelaxant responses evoked by ACh result from the release of relaxing factors following stimulation of muscarinic receptors on the endothelium (Furchgott, 1983). In conduit arteries, NO is the principle endothelium-derived relaxing factor released by these agonists (Huang et al., 1995), whereas in smaller arteries, both NO and EDHF may contribute (Shimokawa et al., 1996). Preliminary studies in our laboratory show that ACh acts by stimulating release of

both NO and an NO-independent factor (possibly EDHF) from the endothelium of human small resistance arteries (McIntyre et al., 1998c).

The lack of response to ACh in large resistance arteries was consistent with a previous investigation (Angus et al., 1992) and could, conceivably, be due to: (i) physical damage to the endothelium; (ii) reduced responsiveness of the smooth muscle to relaxant factors; and/or (iii) absence of muscarinic receptors from the endothelial cell. Damage to the endothelium, as a result of either vessel preparation or cardiovascular disease, is unlikely; immunohistochemistry demonstrated an intact endothelial layer in large resistance arteries, while responses to other endothelium-dependent relaxants (BK, A23187) were similar to those obtained in small resistance arteries. Similarly, although diabetes (Chan et al., 2000) and glaucoma (Buckley et al., 1997) are associated with abnormal endothelial function in some anatomical territories, the lack of response to ACh in large resistance arteries cannot be attributed to the presence of these conditions in some of the biopsy donors. Firstly, there were no differences in response to ACh in large resistance arteries obtained from healthy controls and those from patients with diabetes or NPG. Secondly, one small and one large resistance artery were isolated from each biopsy and there was no evidence of endothelial dysfunction in any of the small resistance arteries. These results are consistent with demonstrations by ourselves (McIntyre et al., 1998a,b; Buckley et al., 1999) and others (Cipolla et al., 1996; Malik et al., 1999) that diabetes and NPG do not alter endothelium-dependent relaxation in human gluteal resistance arteries. Reduced responsiveness of the smooth muscle to relaxing factors is also unlikely to account for lack of response to ACh in large resistance arteries given that BK- and A23187-mediated relaxation responses were similar to those obtained in small resistance arteries. Furthermore, SIN-1-mediated relaxation was intact, indicating that the smooth muscle was able to relax in response to NO. These results are consistent with the lack of response to ACh resulting from an absence of muscarinic receptors from the endothelial cells. The failure of these vessels to contract in response to high concentrations of ACh (Fig. 3b) suggests that muscarinic receptors are also absent from the smooth muscle cells. Therefore, lack of relaxation to ACh is not the result of contractile responses predominating over relaxation. This situation contrasts with BK (Fig. 3a), which did produce a contraction at high-concentrations. This is unsurprising as receptor heterogeneity is well described in the endothelium (Vanhoutte, 1989) and our conclusions correspond with those of Angus et al. (1992) who reported that large subcutaneous resistance arteries, which did not respond to ACh, would relax when exposed to substance P. In order to demonstrate unequivocally that muscarinic receptors are absent from the large resistance arteries and veins, direct investigation would be required using, for example, *in situ* hybridisation and/or autoradiography.

The functional responses of subcutaneous veins were evaluated, under identical conditions, to enable comparison with small and large resistance arteries. Immunohistochemistry confirmed that the veins had an intact endothelium, suggesting that their poor responsiveness to endothelium-dependent dilators is indicative of endothelium-dependent relaxation being less important in veins than arteries. It is possible that some endothelial cell function would be evident under more physiological conditions and investigations designed to assess venous function *in vitro* should take this into account. However, these results are consistent with data obtained using large systemic (saphenous, mammary, femoral, pulmonary, splenic) veins (DeMey and Vanhoutte, 1982; Vanhoutte and Miller, 1985; Luscher et al., 1988). The failure of ACh to evoke relaxation may be due to a lack of the necessary receptors, but the small response to BK and the variable response to A23187 suggest that eNOS activity may be less significant in subcutaneous veins. This contrasts with some systemic veins (saphenous, mammary), which, despite responding poorly to ACh, relax significantly in response to A23187 (Luscher et al., 1988) although there is evidence of anatomical variation in this response (Luscher et al., 1988). A similar variation may account for the heterogeneity of the response to A23187 in the veins used in the present study. As with systemic veins (Luscher et al., 1988), exposure to SIN-1 caused complete relaxation of human subcutaneous veins. The sensitivity to this nitric oxide donor, however, was lower than that of small resistance arteries, further suggesting that the action of NO is less important in veins than arteries from human subcutaneous fat.

Unlike conduit arteries (Martin et al., 1986), basal release of endothelium-derived relaxing factors does not appear to modulate NA-mediated contraction in small resistance arteries as responses to this agonist were not enhanced in denuded vessels (Buckley et al., 1999). The larger maximum response to NA in large resistance arteries compared with small resistance arteries and veins is likely to be the result of size differences, rather than variations in receptor expression, as a similar difference was seen with potassium. Furthermore, the relative size of the response to NA was similar in these vessels when expressed as a percentage of the response to NA-K to correct for variations in vessel size (Lew and Angus, 1992). In contrast, the tendency towards greater amplitude (E_{\max}) and sensitivity (pD_2) of response to ET-1 in large resistance arteries and veins is not simply due to size variations. This may suggest heterogeneity in ET receptor populations, but, alternatively, it is possible that the inhibitory role of the endothelium on ET-1-mediated contraction that has been reported in human small resistance arteries (Buckley et al., 1999) is absent from large resistance arteries and veins.

The demonstration of heterogeneous endothelial cell function in small and large arteries from subcutaneous fat biopsies has implications for investigations using this source of tissue. This is emphasised by the fact that the results

obtained in these subcutaneous vessels are not ubiquitous. For example, our own (unpublished) observations are that large (first order), medium (second order) and small (third order) rat mesenteric arteries all relax in response to ACh. Therefore, it is likely that functional variations exist in similar sized vessels from different vascular territories and care must be taken not to extrapolate the current results to other systems. Furthermore, while the present study distinguishes between function in "small" ($<200\text{ }\mu\text{m}$) and "large" ($\sim 500\text{ }\mu\text{m}$) resistance arteries, it has not been possible to determine an exact demarcation between arteries which respond to ACh and those which do not. The importance of this distinction is highlighted by studies in which ACh-mediated relaxation is impaired in patients, whereas responses to other dilators (BK, substance P, sodium nitroprusside) are unaltered (McNally et al., 1994; Lewis et al., 1998). Studies of resistance artery function often use vessels with a mean internal diameter between 200 and 300 μm . Inclusion of a small number of arteries, which do not respond to ACh, could have a significant effect on the results of such investigations.

In conclusion, this investigation has provided criteria for distinguishing between large and small resistance arteries, and small veins, obtained from human gluteal fat on the basis of physical appearance, differences in response to transmural stretch and functional reactivity. It was demonstrated that heterogeneity of ACh-mediated, endothelium-dependent relaxation in resistance arteries was probably the result of large resistance arteries lacking muscarinic receptors on the endothelium. This suggests variation in endothelial cell receptor populations as resistance artery diameter increases and indicates the importance of confirming that arteries isolated from subcutaneous fat, particularly those with internal diameter $>200\text{ }\mu\text{m}$, are responsive to both ACh and another (e.g., BK, substance P or A23187) endothelium-dependent vasorelaxant.

5. Summary

The nature of, and mechanisms responsible for, functional heterogeneity between small (internal diameter $\leq 200\text{ }\mu\text{m}$) and large (internal diameter $\sim 500\text{ }\mu\text{m}$) resistance arteries obtained from human subcutaneous fat have not been elucidated. This investigation aimed to identify the cause(s) of these functional differences and provide criteria for distinguishing between the different vessel types isolated from biopsies of human gluteal fat. Small ($n=11$) and large ($n=11$) resistance arteries and veins ($n=5$) were isolated from 14 gluteal biopsies. Using small vessel myography, isometric responses were obtained to cumulative additions of vasoconstrictors (NA, potassium and ET-1) and endothelium-dependent (ACh, BK and the calcium ionophore, A23187) and -independent (SIN-1) vasodilators. Immunohistochemical analysis confirmed that all vessels had an intact endothelium. Responses to vasoconstrictor agonists

were similar in large and small resistance arteries and veins. Small resistance arteries relaxed in response to ACh (E_{max} $95.74 \pm 1.86\%$), BK ($88.35 \pm 4.76\%$), A23187 ($76.00 \pm 11.52\%$) and SIN-1 ($98.70 \pm 2.64\%$). Large resistance arteries were selectively unresponsive to ACh, despite exhibiting similar responses to BK ($78.67 \pm 3.92\%$; $P=.26$), A23187 ($67.00 \pm 8.36\%$; $P=.84$) and SIN-1 ($100.15 \pm 2.75\%$; $P=.99$). In contrast, only SIN-1 caused equivalent relaxation ($109.00 \pm 18.52\%$; $P=.69$) in veins. These results indicate a heterogeneity in endothelial cell function between large and small resistance arteries from human gluteal fat, which may be the result of variations in receptor population. This emphasises the need to ensure careful selection of arteries from this source, which can be achieved using physical and functional criteria identified, when performing functional investigations.

Acknowledgments

C.H.B. was supported by the Royal National Institute for the Blind (London, UK), C.A.M. by a British Diabetic Association project grant and P.W.F.H. by a project grant from the British Heart Foundation. Our thanks to Mr. A. Elliott and Drs. E. Henry, R. Andrews, J.A. McKnight, R. Parris, C. O'Brien and B.R. Walker for patient recruitment and gluteal biopsy.

References

- Aalkjaer, C., Danielsen, H., Johannesen, P., Pedersen, E.B., Rasmussen, A., Mulvany, M.J., 1985. Abnormal vascular function and morphology in pre-eclampsia — a study of isolated resistance vessels. *Clin. Sci.* 69 (4), 477–482.
- Angus, J.A., Jennings, G.L., Sudhir, K., 1992. Enhanced contraction to noradrenaline, serotonin, and nerve stimulation but normal endothelium-derived relaxing factor response in skin small arteries in human primary hypertension. *Clin. Exp. Pharmacol. Physiol.* 19 (Suppl. 19), 39–47.
- Angus, J.A., Ferrier, C.P., Sudhir, K., Kaye, D., Jennings, G., 1993. Impaired contraction and relaxation in skin resistance arteries from patients with congestive heart failure. *Cardiovasc. Res.* 27, 204–210.
- Bell, G.H., Emslie-Smith, D., 1980. *Textbook of physiology: The Circulation*, 10th ed. Livingstone, London, pp. 74–175.
- Buckley, C.H., Hadoke, P.W.F., O'Brien, C.J., 1997. Review article. The use of ocular arteries in vitro to define the pathology of vascular changes in glaucoma. *Br. J. Ophthalmol.* 81, 599–607.
- Buckley, C., Hadoke, P.W.F., Henry, E., O'Brien, C.J., 1999. Functional responses of subcutaneous resistance arteries isolated from patients with normal pressure glaucoma. *Br. J. Pharmacol.* 128 (Proc. Suppl.), P53: (Abstract).
- Chan, N.N., Vallance, P., Colhoun, H.M., 2000. Nitric oxide and vascular responses in type I diabetes. *Diabetologia* 43, 137–147.
- Cipolla, M.J., Harker, C.T., Porter, J.M., 1996. Endothelial function and adrenergic reactivity in human type II diabetic resistance arteries. *J. Vasc. Surg.* 23, 940–949.
- DeMey, J.G., Vanhoutte, P.M., 1982. Heterogeneous behaviour of the canine arterial and venous wall: importance of the endothelium. *Circ. Res.* 51, 439–447.

- Furchgott, R.F., 1983. Role of endothelium in responses of vascular smooth muscle. *Circ. Res.* 53, 557–573.
- Huang, P.L., Huang, Z., Mashimo, H., Bloch, K.D., Moskowitz, J.A., Bevan, J.A., Fishman, M.C., 1995. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 377, 239–242.
- Lew, M.J., Angus, J.A., 1992. Wall thickness to lumen-diameter ratios of arteries from SHR and WKY: comparison of pressurised and wire-mounted preparations. *J. Vasc. Res.* 29, 435–442.
- Lewis, T.V., Cooper, B.A., Dart, A.M., Chin-Dusting, J.P.F., 1998. Responses to endothelium-dependent agonists in subcutaneous arteries excised from hypercholesterolaemic men. *Br. J. Pharmacol.* 124 (1), 222–228.
- Luscher, T.F., Diederich, D., Siebenmann, R., Lehmann, K., Stulz, P., von Segesser, L., Yang, Z., Turina, M., Gradel, E., Weber, E., Buhler, F.R., 1988. Difference between endothelium-dependent relaxation in arterial and in venous coronary bypass grafts. *N. Engl. J. Med.* 319, 462–467.
- Malik, R.A., Paniagua, O., Shaw, L., Austin, C., Heagerty, A.M., 1999. Resistance vessel function and structure in type 1 diabetes. *Diabetologia* 42 (Suppl. 1), A75 (Abstract).
- Martin, W., Furchgott, R.F., Villani, G.M., Jothianandan, D., 1986. Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. *J. Pharmacol. Exp. Ther.* 237, 529–538.
- McIntyre, C.A., Hadoke, P.W.F., Williams, B.C., Lindsay, R.M., Elliot, J.A., McKnight, J.A., 1998a. Endothelial and vascular smooth muscle cell function in resistance arteries isolated from patients with insulin-dependent diabetes mellitus. *Diabetic Med.* 15 (Suppl. 2), P7 (Abstract).
- McIntyre, C.A., Williams, B.C., Andrews, R.C., McKnight, J.A., Walker, B.R., Hadoke, P.W.F., 1998b. Impaired receptor-independent vasoconstriction in resistance arteries isolated from patients with non-insulin-dependent diabetes mellitus. *Diabetic Med.* 15 (Suppl. 2), P6 (Abstract).
- McIntyre, C.A., Andrews, R.C., Elliot, A., Gray, G.A., Williams, B.C., McKnight, J., Walker, B.R., Hadoke, P.W.F., 1998c. Endothelium-derived hyperpolarising factor mediates to a large extent acetylcholine-induced relaxation of human subcutaneous resistance arteries. *Br. J. Pharmacol.* 125 (Proc. Suppl.), 16P (Abstract).
- McNally, P.C., Watt, P.A.C., Rimmer, T., Burden, A.C., Hearnshaw, J.R., Thurston, H., 1994. Impaired contraction and endothelium-dependent relaxation in isolated resistance vessels from patients with insulin-dependent diabetes. *Clin. Sci.* 87, 31–36.
- Mulvany, M.J., Aalkjaer, C., 1990. Structure and function of small arteries. *Physiol. Rev.* 70 (4), 921–961.
- Mulvany, M.J., Halpern, W., 1977. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.* 41, 19–25.
- Ohkubo, H., Chiba, S., 1988. Regional differences of vascular sensitivities in canine long posterior ciliary arteries. *Curr. Eye Res.* 7, 457–463.
- Shimokawa, H., Yasutake, H., Fujii, K., Owada, M.K., Nakaike, R., Fukumoto, Y., Takayanagi, T., Nagao, T., Egashira, K., Fujishima, M., Takeshita, A., 1996. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J. Cardiovasc. Pharmacol.* 28 (5), 703–711.
- Smith, P.J.W., Ferro, C.J., McQueen, D.S., Webb, D.J., 1999. Impaired cholinergic dilator responses of resistance arteries isolated from patients with Raynaud's disease. *Br. J. Clin. Pharmacol.* 47 (5), 507–513.
- Vanhoutte, P.M., 1989. Endothelium and control of vascular function. State of the art lecture. *Hypertension* 13, 658–667.
- Vanhoutte, P.M., Miller, V.M., 1985. Heterogeneity of endothelium-dependent responses in mammalian blood vessels. *J. Cardiovasc. Pharmacol.* 7 (Suppl. 3), S12–S23.

Patrick Hadoke

From: "Editorial Office" <editorial@portlandpress.com>
To: <Patrick.Hadoke@ed.ac.uk>
Sent: 07 June 2002 16:33
Subject: Copyright permission

7 June 2002

Dear Ms McIntyre,

Thank you for contacting Portland Press to apply for permission to include the following paper in your thesis:

C.-A. McIntyre et al. Clinical Science (2001) 100, 311-318

I am happy to grant you permission to reproduce the paper in your thesis provided that it is not used for commercial purposes and a credit line in the following format is used:

"Reproduced with permission, from Author(s), (year of publication), (Journal title), (volume number), (page range). © the Biochemical Society and the Medical Research Society"

I wish you luck with the completion of your studies.

Yours sincerely,

Audrey McCulloch
Publications Admin Manager
editorial@portlandpress.com

Portland Press Limited

All e-mails sent to or from this address are separately archived and delivered by the Portland Press Corporate e-mail system. The archive, which is permanent, is subject to review by authorised personnel.

Selective enhancement of sensitivity to endothelin-1 despite normal endothelium-dependent relaxation in subcutaneous resistance arteries isolated from patients with Type 1 diabetes

Carol-Ann McINTYRE*, Patrick W. F. HADOKE*, Brent C. WILLIAMS*,
R. Mark LINDSAY†, Andrew I. ELLIOTT† and John A. McKNIGHT†

*Department of Medical Sciences, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, Scotland, U.K., and †Metabolic Unit, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, Scotland, U.K.

A B S T R A C T

Type 1 diabetes mellitus is associated with abnormal vascular function, but few studies have documented its effects on human resistance arteries. This study aimed to determine whether endothelial cell and smooth muscle cell function was impaired in resistance arteries isolated from patients with this condition. Biopsies of subcutaneous gluteal fat were taken from 12 patients with Type 1 diabetes (age 32.3 ± 1.9 years; duration of diabetes 13.9 ± 2.5 years) and 12 matched controls (age 31.5 ± 2.2 years). Levels of glycosylated haemoglobin were higher ($P < 0.0001$) in patients ($9.38 \pm 0.35\%$) than in controls ($5.48 \pm 0.11\%$), but most (11 out of 12) patients showed no evidence of microvascular disease. Small resistance arteries were isolated from the biopsies, and isometric responses to vasoconstrictors and vasodilators were measured in a small-vessel myograph. The magnitude and sensitivity of responses to noradrenaline and potassium were not different in diabetic patients compared with controls. In contrast, the sensitivity (pD_2 ; negative logarithm of the concentration of the vasoconstrictor required to produce 50% of the maximum effect), but not the magnitude, of contraction in response to endothelin-1 in vessels from patients (8.87 ± 0.12) was significantly ($P = 0.02$) greater than in those from controls (8.40 ± 0.13). Endothelium-dependent (acetylcholine, bradykinin, A23187) and -independent (3'-morpholinovinylsindonimine) relaxation responses were unaltered in patients with Type 1 diabetes. These results suggest a selective alteration in receptor activity in the endothelium, and contrast strikingly with the considerable evidence of impaired endothelium-dependent relaxation in Type 1 diabetes. The present study indicates, therefore, that endothelial cell function is largely maintained in resistance arteries from patients with well controlled Type 1 diabetes. The increased response to endothelin-1 supports the possibility that more significant abnormalities would be evident in patients with severe microvascular complications.

Key words: endothelium, human resistance arteries, Type 1 diabetes, vasoconstriction, vasorelaxation.

Abbreviations: ACh, acetylcholine; ET-1, endothelin-1; NA, noradrenaline; pD_2 , negative logarithm of the concentration of vasoconstrictor required to produce 50% of the maximum effect; PSS, physiological salt solution; KPSS, PSS in which NaCl has been replaced by an equimolar concentration of KCl; SIN-1, 3'-morpholinovinylsindonimine.

There is compelling evidence to indicate that the ability of the endothelium to mediate vascular relaxation is impaired in large conduit arteries from patients and animals with Type I diabetes mellitus [1,2]. Similarly, contractile function may also be altered in these vessels, although the nature of this alteration remains controversial. These abnormalities have serious consequences, as impaired function of conduit arteries has been implicated in the increased risk of atheroma in patients with Type I diabetes [2].

A significant proportion of the morbidity and mortality associated with Type I diabetes results from complications such as retinopathy, nephropathy and neuropathy [3]. There is considerable evidence that, as with atheroma, these microvascular complications are the result of vascular dysfunction [4]. There have been relatively few studies of microvascular function in patients with Type I diabetes, however, and consequently both the nature and the causes of any dysfunction remain controversial [5]. It seems unlikely that Type I diabetes would produce the same pathophysiological changes in conduit and resistance arteries, as it is well established that these vessels are functionally distinct. Receptor populations vary in arteries of different anatomical origin [6] and, perhaps more significantly, the factors that contribute to endothelium-dependent relaxation differ in large compared with small vessels. Endothelium-dependent relaxation is mediated almost exclusively by nitric oxide (NO) in conduit arteries [7], whereas the contribution of a putative endothelium-derived hyperpolarizing factor becomes increasingly significant as the diameter of the vessel diminishes [8].

The few studies of vascular function using resistance arteries isolated from either patients with Type I diabetes [9] or animal models of this condition [10] have suggested impaired endothelium-dependent relaxation. Surprisingly, however, the dysfunction detected in human arteries [9] was specific for responses to acetylcholine (ACh), a conclusion that has been challenged by preliminary data from a more recent study [11]. The impact of Type I diabetes on contractile function in resistance arteries is also controversial: contraction of human resistance arteries appeared to be unchanged or impaired in patients with diabetes [9,11], whereas responses to noradrenaline (NA) were enhanced in mesenteric arteries from diabetic rats [10]. The present investigation aimed to clarify the impact of Type I diabetes on resistance artery function by studying both contractile and relaxant responses in resistance arteries taken from patients with this condition. Endothelin-1 (ET-1) was included in the investigation, as this potent vasoconstrictor, which can be released from damaged endothelial cells, has been implicated in the pathogenesis of vascular complications in Type I diabetes [12], but its effects have not been studied

previously in resistance arteries from patients with this condition.

METHODS

Subjects

Twelve patients (seven male, five female) with Type I diabetes, and 12 age- and sex-matched healthy controls, with no history of diabetes, were recruited for the study. Patients were selected on the basis that they had no history of cardiovascular disease and were taking no major drugs other than insulin. Controls were recruited from the general population and had no history of cardiovascular disease or diabetes. Six patients and four controls were smokers, and three patients were also taking oral contraceptives. All patients provided informed written consent, and the study protocol was approved by the Lothian Research Ethics Committee. For each subject, blood pressure was measured three times (sitting, left arm; sitting, right arm; standing, left arm) to obtain mean values for systolic and diastolic pressure. The presence of retinopathy was assessed using dilated pupil ophthalmoscopy, while neuropathy was tested by measuring vibration in the toe. The presence of microalbuminuria was assessed in patients by calculation of the albumin/creatinine ratio.

Preparation of arteries

A biopsy of skin and subcutaneous fat (approx. 2 cm long \times 1 cm \times 1 cm) was taken from the gluteal region under local anaesthesia (5 ml of 2% lignocaine hydrochloride; Astra, King's Langley, Herts., U.K.). Resistance arteries were dissected from the biopsy and mounted as ring preparations on two 40 μ m stainless steel wires in a small-vessel myograph (J. P. Trading, Aarhus, Denmark). One of these wires was fixed to a movable micrometer, while the other was attached to an isometric force transducer. The vessels were immersed in physiological salt solution (PSS; composition (mM): NaCl, 119; KCl, 4.7; CaCl₂, 2.5; MgSO₄ \cdot 7H₂O, 1.17; KH₂PO₄, 1.18; NaHCO₃, 25; K₂EDTA, 0.026; D-glucose, 5.5), which was maintained at 37 °C and perfused continuously with 95% O₂/5% CO₂. The length of each vessel segment was measured using a travelling micrometer eyepiece attached to a light microscope. Following an equilibration period (30 min), the length-tension characteristics were determined by subjecting each vessel to incremental stretches and applying the La Place equation as described previously [13]. This calculates the internal diameter (l_{100}) that the vessel would have *in vivo* when relaxed and subjected to a pressure of 13.3 kPa (100 mmHg). The vessels were then stretched to their optimum resting setting ($0.9l_{100}$) [10].

Table 1 Characteristics of control subjects and patients with Type 1 diabetes

Values are mean \pm S.E.M. All values are for 12 observations, except when indicated otherwise by *n* values quoted in parentheses, and were compared using Student's unpaired *t*-test.

Parameter	Controls	Diabetic patients	<i>P</i>
Age (years)	31.5 \pm 2.2	32.3 \pm 1.9	0.78
Duration of Type 1 diabetes (years)	—	13.9 \pm 2.5	—
Blood pressure (mmHg)			
Systolic	122 \pm 3 (10)	121 \pm 3	0.78
Diastolic	79 \pm 2 (10)	76 \pm 2	0.51
Glucose (mmol/l)	4.85 \pm 0.07 (11)	13.84 \pm 1.84 (10)	< 0.0001
Glycosylated haemoglobin (%)	5.48 \pm 0.11	9.38 \pm 0.35	< 0.000001
Serum cholesterol (mmol/l)	4.55 \pm 0.36 (11)	5.25 \pm 0.28	0.13
Serum triacylglycerol (mmol/l)	1.17 \pm 0.19 (11)	1.72 \pm 0.38	0.22
Serum creatinine (μ mol/l)	75.6 \pm 3.6 (11)	68.8 \pm 2.4	0.12
Serum albumin (g/l)	42.08 \pm 0.63	38.4 \pm 3.3	0.38
Urea (mmol/l)	3.98 \pm 0.34	4.23 \pm 0.19	0.52

Protocol

Arterial rings were equilibrated at their optimum resting setting for 30 min, and were then subjected to a standard start procedure. This consisted of five consecutive applications of the vasoconstrictors NA and potassium (K^+). After each exposure, the artery was washed several times with PSS and the responses were allowed to return to baseline. The first, second and fifth contractions were produced using NA/ K^+ [a 125 mM K^+ solution (KPSS; made by equimolar substitution of KCl for NaCl in PSS) containing 10 μ M NA]. The third contraction was produced using KPSS, while the fourth was obtained using 10 μ M NA. This procedure confirmed the viability of each artery, and demonstrated the reproducibility of the vasoconstrictor responses.

Cumulative concentration-response curves were obtained using the receptor-dependent vasoconstrictors NA (1 nM–30 μ M) and ET-1 (0.01 nM–0.3 μ M) and using elevated K^+ concentrations (10–125 mM), which cause receptor-independent contraction by depolarizing the vascular smooth muscle. Responses to vasodilators were obtained following contraction of the artery with a submaximal concentration of NA (3 μ M). Cumulative concentration-response curves were also obtained for the receptor-dependent, endothelium-dependent agonists ACh (1 nM–30 μ M) and bradykinin (0.1 nM–3 μ M), the receptor-independent, endothelium-dependent calcium ionophore A23187 (1 nM–3 μ M) and the endothelium-independent nitric oxide donor 3'-morpholinodisynonimine (SIN-1; 1 nM–30 μ M).

Statistics

All values shown are mean \pm S.E.M. for *n* experiments, where *n* represents the number of subjects. Contractile responses are expressed in mN/mm, and also as a percentage of the maximum response to the third stimulation with NA/ K^+ (% NA/ K^+). Relaxation is

expressed as a percentage of the precontraction with NA. Sensitivity values were obtained by fitting the Hill equation to the data using curve-fitting software (Fig P; Biosoft, Cambridge, U.K.). These are expressed as the negative logarithm of the concentration of the agonist required to produce 50% of the maximum effect (pD_2 for vasoconstrictors; $-\log IC_{50}$ for vasodilators). Comparisons of maximum responses and sensitivities were achieved using Student's unpaired *t*-test, and significance was assumed when *P* < 0.05.

Drugs

All salts were obtained from BDH (Poole, Dorset, U.K.). NA hydrochloride, ACh chloride, bradykinin acetate salt and A23187 free acid were obtained from Sigma (Poole, Dorset, U.K.). ET-1 and SIN-1 were obtained from Alexis (Nottingham, U.K.). Stock solutions (1 mM) were prepared in distilled water, except for ET-1, which was initially dissolved in 50% (v/v) methanol to give a 10 μ M stock solution, and A23187, which was initially dissolved in ethanol to give a 0.1 mM stock solution. Subsequent dilutions were made in distilled water, and final bath concentrations of methanol and ethanol did not exceed 1.5% and 3% (v/v) respectively. Stock solutions were frozen as 1 ml aliquots at -20°C and thawed as required. Any residual solution was discarded at the end of the experiment.

RESULTS

The characteristics of the subjects recruited for this investigation are given in Table 1. Eleven of the patients with diabetes had no history of macrovascular or microvascular complications, nor any evidence of retinopathy or neuropathy. One patient had proliferative retinopathy, but no elevation of the albumin/creatinine

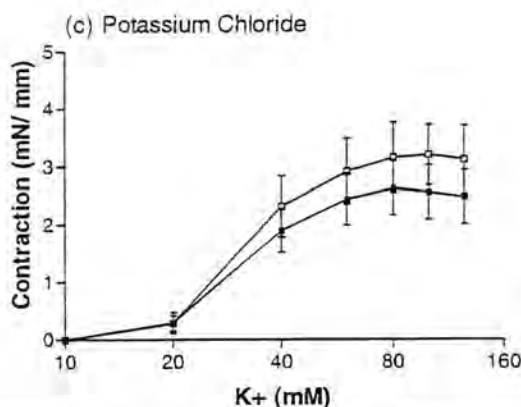
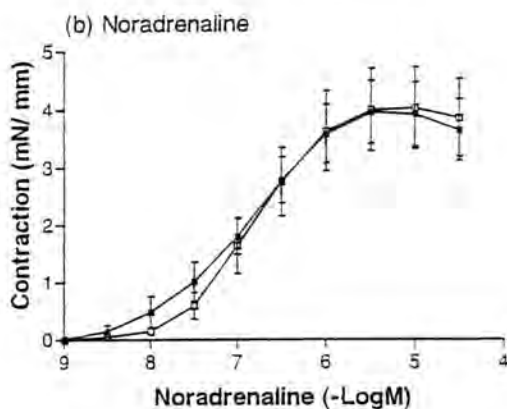
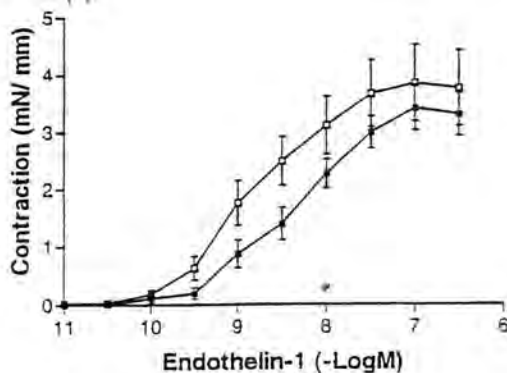


Figure 1 Cumulative concentration-response curves to the vasoconstrictor agonists (a) ET-1, (b) NA and (c) KCl obtained using arteries from control subjects (■) and patients with Type I diabetes (□)

Values are means \pm S.E.M. ($n = 12$).

ratio (functional responses of arteries from this individual were not different from those from the other 11 patients). Urinary albumin/creatinine ratios (1.11 ± 0.26 mg/mmol; $n = 12$) were within the normal range (0.0–3.5 mg/mmol), indicating normal renal function.

Gluteal resistance arteries isolated from patients and

with Type I diabetes and controls in response to NA, potassium (K^+) and ET-1

Results are expressed as a percentage of the maximum response to NA/ K^+ . All values are means \pm S.E.M. ($n = 12$), and were compared using Student's unpaired t -test.

Vasoconstrictor	Maximum contraction (% of contraction to NA/ K^+)		<i>P</i>
	Controls	Patients	
NA	115.4 ± 3.2	108.3 ± 2.0	0.09
K^+	71.9 ± 6.7	84.1 ± 7.8	0.22
ET-1	103.7 ± 5.9	104.5 ± 6.03	0.92

controls were of a similar size (mean internal diameter 224 ± 22 μ m and 195 ± 9 μ m respectively; $P = 0.24$). All arteries produced concentration-dependent contractions in response to the three vasoconstrictors. The sensitivity (pD_2) to ET-1 (Figure 1a) in arteries from patients with diabetes (8.87 ± 0.12) was significantly ($P = 0.02$) greater than that in arteries from controls (8.40 ± 0.12), but the maximum contractions were similar (3.85 ± 0.66 and 3.39 ± 0.39 mN/mm respectively; $P = 0.56$). In contrast, sensitivities (pD_2 values) of arteries from patients to NA (6.73 ± 0.11) and KPSS (1.50 ± 0.04) were similar to those of arteries from controls [6.98 ± 0.14 ($P = 0.19$) and 1.47 ± 0.03 ($P = 0.55$) respectively]. Maximum contractile responses to NA (Figure 1b) and KPSS (Figure 1c) were also similar in patient (4.06 ± 0.71 and 3.26 ± 0.61 mN/mm respectively) and control [2.67 ± 0.47 mN/mm ($P = 0.46$) and 3.39 ± 0.39 mN/mm ($P = 0.56$) respectively] arteries. No differences were detected between control and diabetic groups when values for maximum contractions were expressed as a percentage of the maximum contraction in response to NA/ K^+ , in order to control for variations in the size of the arterial rings (Table 2).

The four vasodilators used all produced concentration-dependent relaxation in arteries precontracted with a submaximal concentration of NA. Responses to vasodilators in arteries from the three diabetic patients taking oral contraceptives were not different from those obtained in arteries from the rest of the patient group. Arteries from control subjects produced near-maximal relaxation in response to ACh ($97.2 \pm 1.0\%$; Figure 2a), bradykinin ($94.3 \pm 2.2\%$, $n = 8$; Figure 2b) and SIN-1 ($93.8 \pm 4.4\%$; Figure 2c). Similar responses were produced in arteries from patients with Type I diabetes [$95.9 \pm 1.8\%$ ($P = 0.50$), $91.7 \pm 2.2\%$ ($n = 9$) ($P = 0.38$) and $95.5 \pm 3.2\%$ ($P = 0.76$) respectively]. A23187 produced a smaller relaxation than the other three vasodilators, and did not achieve a definite maximum in the concentration range used (Figure 2d). However, the peak relaxation was similar ($P = 0.21$) in arteries from patients

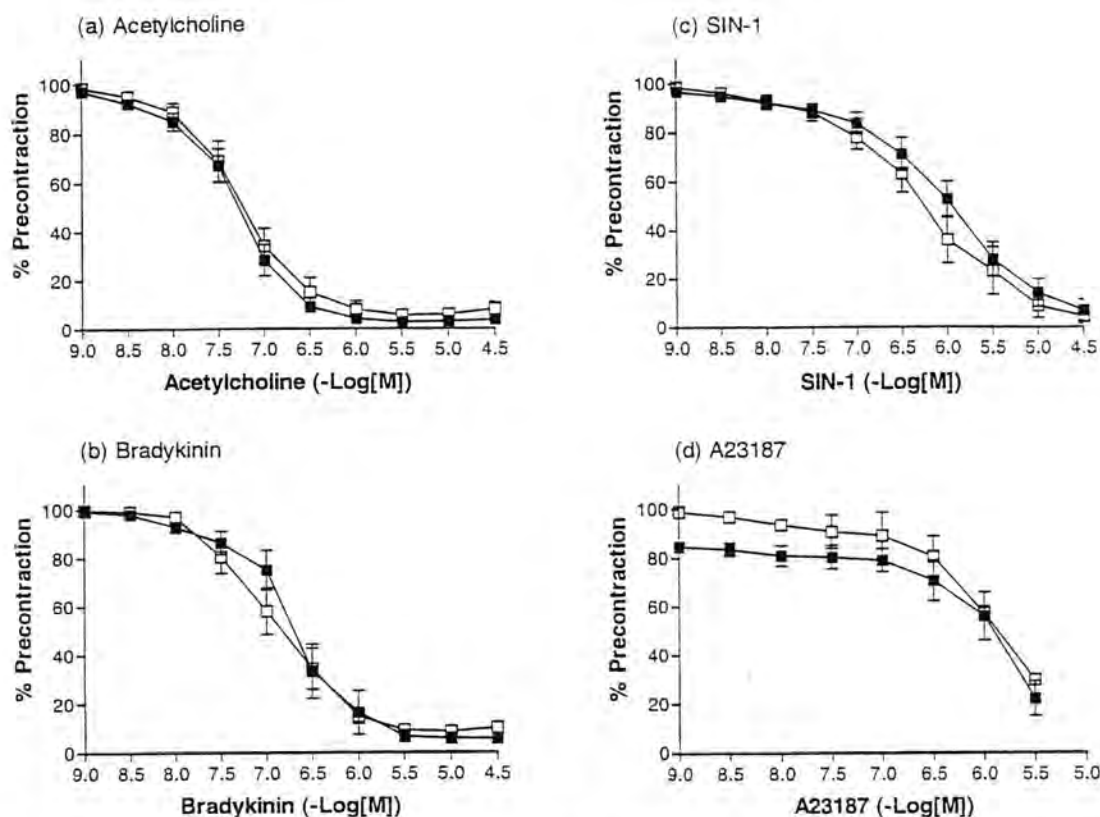


Figure 2 Cumulative concentration-response curves to the vasodilator agonists (a) ACh, (b) bradykinin, (c) SIN-1 and (d) A23187 obtained using arteries from control subjects (■) and patients with Type I diabetes (□)

Values are means \pm S.E.M. for (a) $n = 12$, (b) $n = 8$, (c) $n = 8-10$, and (d) $n = 11-12$.

Table 3 Sensitivity ($-\log IC_{50}$ values) from concentration-response curves obtained to various vasodilatory agonists

All values are mean \pm S.E.M. for $n = 12$, unless indicated otherwise by numbers in parentheses, and were compared using Student's unpaired t -test. Sensitivity values for A23187 are only approximate, as the response to this agonist did not reach a maximum in the concentration range used.

Vasodilator	Sensitivity ($-\log IC_{50}$)		P
	Controls	Patients	
ACh	7.30 ± 0.09	7.23 ± 0.12	0.63
Bradykinin	7.69 ± 0.15 (8)	7.95 ± 0.15 (9)	0.29
A23187	5.96 ± 0.18 (9)	6.10 ± 0.16 (6)	0.61
SIN-1	5.87 ± 0.21	6.22 ± 0.22	0.26

with diabetes (72.0 ± 6.8 , $n = 7$) and controls (81.5 ± 3.4 , $n = 9$). The sensitivity ($-\log IC_{50}$) of relaxation to ACh, bradykinin, SIN-1 and A23187 in diabetic patients was similar to that in controls (Table 3).

Functional responses to vasodilators (maximum relaxation and $-\log IC_{50}$) and vasoconstrictors (maximum contraction and pD_2) did not correlate with age, duration of diabetes, plasma glucose or levels of glycosylated haemoglobin.

DISCUSSION

This investigation has made two striking observations in resistance arteries from patients with Type I diabetes: (i) a selective enhancement of ET-1-induced contraction, and (ii) normal endothelium-dependent (and endothelium-independent) relaxation. This indication of a limited, selective abnormality in endothelial cell function contrasts directly with the dramatic impairment of endothelium-dependent relaxation generally reported in Type I diabetes. These results indicate that endothelial cell function is largely maintained in subcutaneous resistance arteries from patients with well controlled Type I diabetes.

Damage to the endothelium often results in increased release of ET-1. Consequently, the elevated plasma [14] and tissue [15] concentrations of this peptide in patients suggest that damage to the endothelium is a feature of Type I diabetes. Similarly, urinary concentrations of ET-1 are elevated [16], and its release from resistance arteries is enhanced [17], in diabetic rats, while increased release of ET-1 possibly contributes to the down-regulation of glomerular ET receptors [18]. The precise role of ET-1 in the development of vascular complications, however, remains unclear. Functional data obtained using arteries

from diabetic rats are contradictory, and there is evidence that the duration of diabetes has a significant impact on both ET-1-mediated contraction and endothelial cell function [12].

The selective enhancement of ET-1-mediated contraction in arteries from patients with Type I diabetes suggests altered function of the ET receptors in the endothelium and/or smooth muscle cells. This contrasts with results from studies using diabetic animals, in which increased ET-1-mediated contraction was just one component of a non-specific enhancement of contractile function, possibly mediated by altered second messenger activity [19,20]. Unaltered responses to NA and KPSS in the present study indicated that vascular remodelling, or a general abnormality in second messenger systems, was not responsible for the increased response to ET-1. Endothelium-derived relaxing factors have a modulatory effect on the ET-1-mediated contraction of human subcutaneous resistance arteries [21]. Consequently, removal of the endothelium results in an increase in sensitivity (but not in maximum contraction) of ~ 10% [21]. The current investigation suggests that this modulatory function of the endothelium has been partially lost in arteries from patients with Type I diabetes. As the ability of the endothelium to mediate relaxation was not impaired, this may suggest down-regulation of endothelial ET_B receptors. This is strikingly similar to results obtained using resistance arteries from patients with hypertension [22] and normal pressure glaucoma [21]. Alternatively, the enhanced ET-1-mediated contraction may be a consequence of up-regulation of ET_A and/or ET_B receptors on the vascular smooth muscle cells. Further studies would be required to identify the exact mechanism(s) responsible for the enhanced response to ET-1.

Our demonstration of unaltered responses to NA is consistent with data obtained in the isolated forearm [23,24] and in resistance arteries from patients with Type I diabetes [11]. The observation of increased angiotensin II-mediated contraction in the latter study supports the contention that contractile abnormalities detected in these arteries are the result of specific alterations in receptor function. This contrasts directly with a previous investigation of resistance artery function in Type I diabetes, which reported a non-specific decrease in contractile function (using NA, angiotensin II and KPSS). It is possible that this impaired contraction was due to a slightly (but not significantly) lower media/lumen ratio in arteries from the diabetic patients [9].

Endothelial cell dysfunction in animal models of diabetes has been indicated by impaired endothelium-dependent relaxation in conduit and resistance arteries [1]. Similar abnormalities have also been reported in the forearm [23] and brachial arteries [25] of patients with Type I diabetes. These results are challenged, however, by similar investigations which have found responses to

endothelium-dependent relaxants to be unchanged [26,27] or even enhanced [28]. The present study used a group of patients with established but generally well controlled diabetes. This compares with a study of conduit arteries *in vivo* [29] which demonstrated that a similar group of patients [with established diabetes (12 years duration) but no microvascular complications] had both structural and functional abnormalities in the aorta and the radial and carotid arteries. The unaltered responses to receptor-dependent (ACh, bradykinin) and receptor-independent (A23187), endothelium-dependent dilators in the present study demonstrate that endothelium-dependent relaxation was not impaired. Furthermore, the maintained response to SIN-1 indicated that the ability of the vascular smooth muscle to relax in response to exogenous NO was similarly unaffected. These results are consistent with studies in which endothelium-dependent relaxation in the human forearm is only impaired in diabetes if patients also exhibit microalbuminuria [24,26,30]. Furthermore, relaxation in response to NO donors (usually sodium nitroprusside) has been shown repeatedly (although not exclusively [11,24]) to be unaltered in patients with this condition [23,26,27,30]. Therefore it is probable that impaired endothelium-derived NO activity in the microvasculature is restricted to patients with evidence of nephropathy. This would correspond with the demonstration that microvascular permeability is only increased in patients with long-standing Type I diabetes if they also have severe microvascular complications [31].

Our results challenge a previous demonstration that ACh-mediated contraction is selectively impaired in resistance arteries isolated from patients with Type I diabetes [9]. The difference between these studies is unlikely to be due to differences in disease duration in the diabetic groups. The duration of diabetes in the group with impaired ACh-mediated relaxation (18 years [9]) was shorter than that in patients who demonstrated normal responses to this agonist (24 years [11]). In contrast with our study, almost all (nine out of ten) of the individuals in the patient group used by McNally *et al.* [9] had evidence of retinopathy, possibly indicating that endothelium-dependent relaxation is only altered in patients with both diabetes and more established microvascular disease. Methodological variations are, however, a more likely reason for these discrepant results. In particular, we have found that some (larger) arteries obtained from subcutaneous biopsies fail to relax in response to ACh, but produce a 90–100% relaxation in response to bradykinin [32]. The inadvertent inclusion of a small number of these arteries in the diabetic group could explain both the specific decrease in ACh-mediated relaxation and the variable size and sensitivity of this response reported in the previous study [9].

In conclusion, the present study has demonstrated an enhanced response to ET-1, despite normal endothelium-

dependent relaxation, in subcutaneous resistance arteries from patients with Type I diabetes. This suggests that endothelial cell function is largely intact in these arteries from patients with established, but well controlled, Type I diabetes. The increased response to ET-1 may result from altered ET_B receptor function on the vascular endothelial cells, and could support the contention that endothelial cell dysfunction is more pronounced in patients with severe microvascular complications. It remains possible that endothelial cell function is altered in resistance arteries in other vascular territories, and also in subcutaneous arteries, from patients with Type I diabetes and associated microvascular complications. Further investigations, such as the recent preliminary report from Malik et al. [11], should confirm the relationship between resistance artery function and the severity of microvascular complications in patients with Type I diabetes.

ACKNOWLEDGMENTS

This work was funded by a British Diabetic Association (Diabetes U.K.) project grant. P.W.F.H. was the recipient of a Department of Medicine (University of Edinburgh) Research Fellowship.

REFERENCES

- Chan, N. N., Vallance, P. and Colhoun, H. M. (2000) Nitric oxide and vascular responses in type I diabetes. *Diabetologia* 43, 137–147.
- Poston, L. and Taylor, P. D. (1995) Endothelium-mediated vascular function in insulin-dependent diabetes mellitus. *Clin. Sci.* 88, 245–255.
- Eurodiab IDDM Complications Study Group (1994) Microvascular and acute complications in IDDM patients: the Eurodiab IDDM Complications Study. *Diabetologia* 36, 454–459.
- Tooke, J. E. (1996) Microvasculature in diabetes. *Cardiovasc. Res.* 32, 764–771.
- DCCT: The Diabetes Control and Complications Trial Research Group (1993) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med.* 329, 977–986.
- Cambridge, D., Whiting, M. V., Butterfield, L. J. and Marston, C. (1995) Vascular 5-HT₁-like receptors mediating vasoconstriction and vasodilatation: their characterisation and distribution in the isolated canine cardiovascular system. *Br. J. Pharmacol.* 114, 961–968.
- Vanhoutte, P. M. (1989) Endothelium and the control of vascular function. *Hypertension* 13, 658–667.
- Shimokawa, H., Yasutake, H., Fujii, K. et al. (1996) The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J. Cardiovasc. Pharmacol.* 28, 703–711.
- McNally, P. C., Watt, P. A. C., Rimmer, T., Burden, A. C., Hearnshaw, J. R. and Thurston, H. (1994) Impaired contraction and endothelium-dependent relaxation in isolated resistance vessels from patients with insulin-dependent diabetes. *Clin. Sci.* 87, 31–36.
- Cameron, N. E. and Cotter, M. A. (1992) Impaired contraction and relaxation in aorta from streptozotocin-diabetic rats: role of polyol pathway. *Diabetologia* 35, 1011–1019.
- Malik, R. A., Paniagua, O., Shaw, L., Austin, C. and Heagerty, A. M. (1999) Resistance vessel function and structure in type I diabetes. *Diabetologia* 42 (Suppl. 1), A75 (Abstract).
- Hopfer, R. L. and Gopalakrishnan, V. (1999) Endothelin: emerging role in diabetic vascular complications. *Diabetologia* 42, 1383–1394.
- Mulvany, M. J. and Halpern, W. (1977) Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.* 41, 19–25.
- Takahashi, K., Ghatei, M. A., Lam, H.-C., O'Halloran, D. J. and Bloom, S. R. (1990) Elevated plasma endothelin in patients with diabetes mellitus. *Diabetologia* 37, 306–310.
- Properzi, G., Terngli, G., Gu, X.-H. et al. (1995) Early increase precedes a depletion of endothelin-1 but not of von Willebrand factor in cutaneous microvessels of diabetic patients. A quantitative immunohistochemical study. *J. Pathol.* 175, 243–252.
- Morabito, E., Corsico, N. and Martelli, E. A. (1995) Endothelin urinary excretion is increased in spontaneously diabetic rats: BB/B. *Pharmacol. Lett.* 56, PL13–PL18.
- Takeda, Y., Miyamami, I., Yaneda, T. and Takeda, R. (1991) Production of endothelin-1 from the mesenteric arteries of streptozotocin-induced diabetic rats. *Life Sci.* 48, 2553–2556.
- Awazu, M., Parker, R. E., Howie, B. R., Ichikawa, I. and Kon, V. (1991) Down-regulation of endothelin-1 receptors by protein kinase C in streptozotocin diabetic rats. *J. Cardiovasc. Pharmacol.* 17 (Suppl. 7), S500–S502.
- White, R. E. and Carrier, G. O. (1990) Vascular contraction induced by activation of membrane calcium ion channels in streptozotocin diabetes. *J. Pharmacol. Exp. Ther.* 253, 1057–1062.
- Hopfer, R. L., McNeill, J. R. and Gopalakrishnan, V. (1999) Endothelin plasma levels and endothelin-1 evoked vascular responses at different temporal stages of diabetes in streptozotocin diabetic rats. *Eur. J. Pharmacol.* 374, 221–227.
- Buckley, C., Hadoke, P. W. F., Henry, E. and O'Brien, C. J. (1999) Functional responses of sub-cutaneous resistance arteries isolated from patients with normal pressure glaucoma. *Br. J. Pharmacol.* 128 (Proc. Suppl.), P53.
- Angus, J. A., Jennings, G. L. and Sudhir, K. (1992) Enhanced contraction to noradrenaline, serotonin and nerve stimulation but normal endothelium-derived relaxing factor response in skin small arteries in human primary hypertension. *Clin. Exp. Pharmacol. Physiol.* 19 (Suppl. 19), 39–47.
- Johnstone, M. T., Creager, S. J., Scales, K. M., Cusco, J. A., Lee, B. K. and Creager, M. A. (1993) Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *Circulation* 88, 2510–2516.
- Calver, A. L., Collier, J. G. and Vallance, P. J. T. (1992) Inhibition and stimulation of nitric oxide synthesis in the fore-arm arterial bed of patients with insulin-dependent diabetes. *J. Clin. Invest.* 90, 2546–2554.
- Zener, B. M., Arcaro, G., Saggiani, F., Rossi, L., Muggeo, M. and Lehi, A. (1995) Non-invasive detection of functional alterations of the arterial wall in IDDM patients with and without microalbuminuria. *Diabetes Care* 18, 975–982.
- Smits, P., Kapma, J. A., Jacobs, M. C., Lutterman, J. and Thien, T. (1993) Endothelium-dependent vascular relaxation in patients with type I diabetes. *Diabetes* 42, 148–153.
- Lambert, J., Aarssen, M., Donker, A. J. and Stenhout, C. D. (1996) Endothelium-dependent and -independent vasodilation of large arteries in normalalbuminaemic insulin-dependent diabetes mellitus. *Arterioscler. Thromb. Vasc. Biol.* 16, 705–711.
- Makimattila, S., Mantysaari, M., Groop, P. H. et al. (1997) Hyper-reactivity to nitrovasodilators in fore-arm vasculature is related to autonomic dysfunction in insulin-dependent diabetes mellitus. *Circulation* 95, 618–625.

- 29 Giannattasio, C., Failla, M., Piperno, A. et al. (1999) Early impairment of large artery structure and function in Type 1 diabetes mellitus. *Diabetologia* 42, 987–994
- 30 Elliot, T. G., Cockcroft, J. R., Groop, P.-H., Earle, K., Morocutti, A. and Viberti, G. C. (1993) Inhibition of nitric oxide synthase in fore-arm vasculature of insulin-dependent diabetic patients: blunted vasoconstriction in patients with microalbuminuria. *Clin. Sci.* 85, 687–693
- 31 Jaap, A. J., Sjöör, A. C. and Tooke, J. E. (1996) Differences in microvascular fluid permeability between long duration type-I diabetic patients with and without significant microangiopathy. *Clin. Sci.* 90, 113–117
- 32 Buckley, C. H., McIntyre, C.-A., and Hadoke, P. W. F. (1999) Heterogeneity of endothelial cell function in vessels isolated from human sub-cutaneous fat. *Br. J. Pharmacol.* 128 (Proc. Suppl.), P263

Received 26 June 2000/26 October 2000; accepted 17 November 2000

Noel Trace

From: Patrick Hadoke [patrick.hadoke@ed.ac.uk]
Sent: 17 October 2002 13:40
To: Noel Trace
Subject: Re: Inclusion of Papers in a thesis

Hi,
with reference to the correspondence below, could I check whether you sent the necessary documentation as we did not receive it.
If possible, could you send the documents again (Fax: 0131 537 1012).
Many thanks,
Patrick Hadoke

— Original Message —

From: Noel Trace
To: 'Patrick Hadoke'
Sent: Tuesday, September 03, 2002 4:10 PM
Subject: RE: Inclusion of Papers in a thesis

Please send me your fax number and I will fax you our stamp of approval.

Kind Regards

Trace Noel
Rights and Reprints Manager

Address:
Nature Publishing Group
Specialist Journals
Brunel Road, Houndmills
Basingstoke
Hampshire
RG21 6XS
Fax: +44 1256 810 526
Direct Tel: +44 1256 302887

NATURE PUBLISHING GROUP granted permission for reproduction of the attached material with the following conditions:

- the source of the material be acknowledged by a full reference to the original article
- permission to reproduce the material is obtained (as far as possible) from the author(s) of the original article(s)

Noel Date: 17/10/02

TRACE NOEL, Rights and Reprints Manager
NATURE PUBLISHING GROUP
Brunel Road, Houndmills, Basingstoke RG21 6XS UK
Tel: +44 (0) 328242 Fax: +44 (0) 1256 810526

— Original Message —

From: Patrick Hadoke [mailto:patrick.hadoke@ed.ac.uk]
Sent: 03 September 2002 16:03
To: t.noel@nature.com
Subject: Inclusion of Papers in a thesis

Dear Dr Noel,
I believe that earlier this year Anna Muir passed on my request (on behalf of my student Carol-Ann McIntyre) for permission to include copies of two papers published in the Brit J Pharmacol in a thesis. Edinburgh University allows this but requires a letter of permission from the journal's publisher.
The papers in question are:

McIntyre et al (2001) Endothelium-derived hyperpolarising factor and potassium use different mechanisms to induce relaxation of human subcutaneous resistance arteries, Br. J. Pharmacol., 133, 902-908

McIntyre et al (1998) Preservation of vascular function in rat mesenteric resistance arteries following cold storage, studied by small vessel myography. Br. J. Pharmacol., 123, 1555-1560.

Could you let me know whether this would be acceptable and let me have a letter (addressed

to Carol-Ann McIntyre) confirming that she has permission?

The thesis is now ready for submission and requires the necessary letter if these two papers are to be included.

Many thanks for your help.

TO: Patrick Hadoke

DISCLAIMER: This e-mail is confidential and should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error please inform the sender and delete it from your mailbox or any other storage mechanism. Neither Macmillan Publishers Limited nor any of its agents accept liability for any statements made which are clearly the sender's own and not expressly made on behalf of Macmillan Publishers Limited or one of its agents. Please note that neither Macmillan Publishers Limited nor any of its agents accept any responsibility for viruses that may be contained in this e-mail or its attachments and it is your responsibility to scan the email and attachments (if any). No contracts may be concluded on behalf of Macmillan Publishers Limited or its agents by means of e-mail communication. Macmillan Publishers Limited Registered in England and Wales with registered number 785998 Registered Office Brunel Road, Houndmills, Basingstoke RG21 6XS

Endothelium-derived hyperpolarizing factor and potassium use different mechanisms to induce relaxation of human subcutaneous resistance arteries

¹C.-A. McIntyre, ¹C.H. Buckley, ¹G.C. Jones, ¹T.C. Sandeep, ¹R.C. Andrews, ²A.I. Elliott, ³G.A. Gray, ¹B.C. Williams, ²J.A. McKnight, ¹B.R. Walker & ^{*,1}P.W.F. Hadoke

¹Department of Medical Sciences, Western General Hospital, University of Edinburgh, Edinburgh; ²Metabolic Unit, Western General Hospital, University of Edinburgh, Edinburgh and ³Department of Biomedical Sciences, Hugh Robson Building, University of Edinburgh, Edinburgh

1 This investigation examined the hypothesis that release of K⁺ accounts for EDHF activity by comparing relaxant responses produced by ACh and KCl in human subcutaneous resistance arteries.

2 Resistance arteries (internal diameter 244 ± 12 µm, n = 48) from human subcutaneous fat biopsies were suspended in a wire myograph. Cumulative concentration-response curves were obtained for ACh (10⁻⁹–3 × 10⁻⁵ M) and KCl (2.5–25 mM) following contraction with noradrenaline (NA; 0.1–3 µM).

3 ACh (E_{max} 99.07 ± 9.61%; –LogIC₅₀ 7.03 ± 0.22; n = 9) and KCl (E_{max} 74.14 ± 5.61%; –LogIC₅₀ 2.12 ± 0.07; n = 10)-induced relaxations were attenuated (P < 0.0001) by removal of the endothelium (E_{max} 8.21 ± 5.39% and 11.56 ± 8.49%, respectively; n = 6–7).

4 Indomethacin (10 µM) did not alter ACh-induced relaxation whereas L-NOARG (100 µM) reduced this response (E_{max} 61.7 ± 3.4%, P < 0.0001; n = 6). The combination of ChTx (50 nM) and apamin (30 nM) attenuated the L-NOARG-insensitive component of ACh-induced relaxation (E_{max} 15.2 ± 10.5%, P < 0.002, n = 6) although these arteries retained the ability to relax in response to 100 µM SIN-1 (E_{max} 127.6 ± 13.0%, n = 3). Exposure to BaCl₂ (30 µM) and Ouabain (1 mM) did not attenuate the L-NOARG resistant component of ACh-mediated relaxation (E_{max} 76.09 ± 8.92, P = 0.16; n = 5).

5 KCl-mediated relaxation was unaffected by L-NOARG + indomethacin (E_{max} 68.1 ± 5.6%, P = 0.33; n = 5) or the combination of L-NOARG/indomethacin/ChTx/apamin (E_{max} 86.61 ± 14.02%, P = 0.35; n = 6). In contrast, the combination of L-NOARG, indomethacin, ouabain and BaCl₂ abolished this response (E_{max} 5.67 ± 2.59%, P < 0.0001, n = 6).

6 The characteristics of KCl-mediated relaxation differed from those of the nitric oxide/prostaglandin-independent component of the response to ACh, and were endothelium-dependent, indicating that K⁺ does not act as an EDHF in human subcutaneous resistance arteries.

British Journal of Pharmacology (2001) 133, 902–908

Keywords: Endothelium-dependent relaxation; endothelium-derived hyperpolarizing factor; nitric oxide; potassium channels; human resistance arteries

Abbreviations: ACh, acetylcholine; BSA, bovine serum albumin; ChTx, charybdotoxin; EDHF, endothelium-derived hyperpolarizing factor; EDTA, ethylene diamine tetraacetic acid; F, Female; KPSS, high potassium physiological salt solution; L-NOARG, N^G-nitro-L-arginine; M, Male; NA, noradrenaline; NO, nitric oxide; PG, prostaglandin; PSS, physiological salt solution; SIN-1, 3'-morpholiniosydnonimine

Introduction

The vascular endothelium modulates agonist-dependent relaxation by releasing substances such as nitric oxide (NO) and prostaglandins (PGs) (Furchgott & Vanhoutte, 1989). In some vessels, particularly those with a small diameter (Shimokawa *et al.*, 1996), a component of the endothelium-dependent relaxation is insensitive to nitric oxide synthase and cyclooxygenase inhibition (Nagao *et al.*, 1992; Brandes *et al.*, 1997). This component appears to be mediated by hyperpolarization of the vascular smooth muscle cells (Brayden, 1990), suggesting the existence of a distinct

endothelium-derived hyperpolarizing factor (EDHF) (Taylor & Weston, 1988; Feletou & Vanhoutte, 1997).

The identity of EDHF has yet to be confirmed, although activity of this factor has been attributed to epoxyeicosatrienoic acids (Hecker *et al.*, 1994), endocannabinoids (Randall *et al.*, 1996), hydrogen peroxide (Matoba *et al.*, 2000) and the presence of myoendothelial gap junctions (Chaytor *et al.*, 1998). A recent study suggested that release of K⁺ into the myoendothelial space accounted for EDHF activity in rat hepatic and mesenteric arteries (Edwards *et al.*, 1998). In this study, EDHF-mediated responses (but not those to exogenous K⁺) were inhibited by using charybdotoxin (ChTx) and apamin to block large (BK_{Ca}) and small (SK_{Ca}) conductance calcium-activated potassium channels on

*Author for correspondence at: Department of Medical Sciences, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU; E-mail: phadoke@srv0.med.ed.ac.uk

the endothelium. In contrast, the combination of barium and ouabain inhibited responses to K^+ as well as to EDHF, suggesting that both EDHF and K^+ cause smooth muscle cell hyperpolarization through activation of inward rectifier potassium channels (K_{IR}) and Na^+/K^+ -ATPases. Subsequent studies have, however, challenged the identification of K^+ as EDHF by demonstrating differences in the characteristics of EDHF and K^+ -induced relaxation in rat mesenteric (Doughty *et al.*, 2000; Lacy *et al.*, 2000), porcine coronary and guinea-pig carotid arteries (Quignard *et al.*, 1999).

Human resistance arteries have been used extensively to examine the cardiovascular defects associated with the development of a variety of different disease processes. A large component of endothelium-dependent relaxation in these arteries is mediated by EDHF (Nakashima *et al.*, 1993; Urakami-Harasawa *et al.*, 1997; Wallerstedt & Bodelsson, 1997) but the mechanism of this response has not been elucidated. This investigation aimed to determine whether K^+ accounted for EDHF activity in human subcutaneous resistance arteries by comparing the NO-independent, PG-independent component of ACh-induced relaxation with relaxant responses produced by exogenous potassium.

Methods

Vessel preparation

Biopsies of gluteal skin and subcutaneous fat (2 cm × 1 cm × 1 cm) were obtained under local anaesthesia (2% lignocaine hydrochloride; Astra, Herts, U.K.) from 26 healthy volunteers (20 Male, six Female; age 46 ± 3 years). Written informed consent and approval from the Lothian Research Ethics Committee were obtained. Each biopsy was immersed immediately in cold (4°C) physiological salt solution (PSS) of the following composition (mM): NaCl 119, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.17, NaHCO₃ 24, KH₂PO₄ 1.18, K₂EDTA 0.026 and D-glucose, 5.5. Dissection of these biopsies provided 48 resistance artery sections (mean internal diameter 244 ± 12 µm) for pharmacological analysis. Ring segments of these arteries, 2 mm in length, were suspended on two 40 µm stainless steel wires in a small vessel myograph for measurement of isometric force. The myograph bath contained PSS maintained at 37°C and perfused with 95% O₂/5% CO₂. Following an equilibration period of 30 min, the resting tension-internal circumference relationship was determined by stepwise radial stretching and the vessels were set to their optimum resting level (0.9 L_{100} , where L_{100} is the internal circumference the vessels would have when relaxed and subjected to a pressure of 100 mmHg; Mulvany & Halpern, 1977). After equilibration for a further 30 min, vessel viability was assessed using a standard start procedure (Aalkjaer *et al.*, 1987). This consisted of five consecutive stimulations lasting 3 min, each followed by a 5 min washout period. The first, second and fifth contractions were produced using a high (125 mM) potassium solution (KPSS; made by equimolar substitution of KCl for NaCl in PSS) containing 10 µM noradrenaline (NA). The third was obtained with NA (10 µM) alone and the fourth with KPSS alone. The functional integrity of the endothelium was assessed by adding ACh (0.1–10 µM) to vessels contracted

with sufficient NA (0.1–3 µM) to produce 60–80% of the response KPSS.

The contribution of EDHF to ACh-mediated relaxation

Sixteen resistance arteries (internal diameter 183 ± 15 µm) from 14 male subjects (age 57 ± 12 years) were used for this part of the investigation. After the standard start procedure, a cumulative concentration-response curve to ACh (0.001–300 µM) was obtained following precontraction with a sub-maximal concentration (0.1–3 µM) of NA (to produce a contraction of ~60–80% the maximum response to KPSS). The artery was washed with PSS (37°C) and the procedure repeated following incubation with either; (a) indomethacin (10 µM for 45 min, $n=6$), (b) N^G-nitro-L-Arginine (L-NOARG; 100 µM for 45 min, $n=6$), or (c) L-NOARG (100 µM for 45 min), plus charybdotoxin (ChTx; 50 nM for 10 min) and apamin (30 nM for 10 min, $n=6$). Arteries were exposed to only one antagonist except for two of those initially incubated with indomethacin which were subsequently exposed to the combination L-NOARG+ChTx+apamin. Three of the arteries incubated with L-NOARG+ChTx+apamin, were also exposed to a single concentration (100 µM) of the exogenous NO donor, 3'-morpholiniosydnonimine (SIN-1) once the concentration-response curve to ACh had been completed.

Comparison of K^+ -induced relaxation with the EDHF-mediated component of ACh-evoked relaxation

Thirty-two resistance arteries (internal diameter 273 ± 14 µm) obtained from 12 subjects (six male, six female; age 32 ± 4 years) were used for this part of the investigation. The endothelium was removed from some arteries by rubbing the luminal surface with a single hair. Cumulative concentration-response curves were obtained using ACh (0.001–300 µM) and KCl (2.5–25 mM), in intact ($n=9-10$) and denuded ($n=6-7$) arteries, after pre-contraction (to produce a contraction of ~60–80% the maximum response to KPSS) with a sub-maximal concentration of NA (0.1–3 µM). Responses to KCl were repeated following incubation with a combination of either (a) L-NOARG (100 µM)+indomethacin (10 µM; 45 min, $n=5$); (b) L-NOARG (100 µM)+indomethacin (10 µM for 45 min) plus charybdotoxin (ChTx; 50 nM for 10 min) and apamin (30 nM for 10 min, $n=6$) or (c) L-NOARG (100 µM)+indomethacin (10 µM for 45 min) plus BaCl₂ (30 µM for 10 min) and ouabain (1 mM for 10 min, $n=6$). Concentration-response curves to ACh were also produced in the arteries exposed to the combinations described for groups (b) and (c).

Drugs

All salts were obtained from BDH Laboratory supplies, (Poole, Dorset, U.K.). All drugs were purchased from Sigma, (Poole, Dorset, U.K.), except for 3'-morpholiniosydnonimine, charybdotoxin and apamin which were obtained from Alexis Corporation Ltd (Nottingham, U.K.). Acetylcholine chloride, ouabain, barium chloride and noradrenaline bitartrate were dissolved in distilled water; indomethacin in 1.5 × 10⁻³ M Na₂CO₃ (final bath concentration of Na₂CO₃ did not exceed 0.015 mM) and apamin in 0.05 M acetic acid (final bath

concentration of acetic acid did not exceed 0.15 mM). Charybdotoxin was dissolved in a Tris buffer (10 mM, pH 7.5) containing 0.1% BSA, 100 mM NaCl and 1 mM EDTA (final bath concentrations of NaCl and EDTA did not exceed 5 and 0.05 mM, respectively). 0.01% BSA was added to the myograph chamber before applying the toxins. Stock solutions were stored at -20°C , thawed as required and subsequent dilutions made in distilled water. The concentrations quoted are final molar concentrations in the organ bath.

Statistics

All values are presented as mean \pm standard error mean (s.e.mean) from n experiments (where n represents the number of subjects). Relaxation responses to ACh and KCl are expressed as a percentage of the initial NA-induced precontraction. The concentration of agonist required to produce 50% of the maximum response (IC_{50}) was obtained by fitting the Hill equation to the data using curve fitting software (Fig. P, Biosoft, Cambridge, U.K.) and is expressed as the negative logarithm of the IC_{50} ($-\log\text{IC}_{50}$). Comparisons of maximum relaxation and $-\log\text{IC}_{50}$ values were made using Student's paired or unpaired t -test, as appropriate, and significance was assumed when $P < 0.05$.

Results

The contribution of EDHF to ACh-induced relaxation

ACh caused approximately 80–100% relaxation in intact human subcutaneous resistance arteries following pre-contraction with a sub-maximal concentration of NA ($0.1\text{--}3\text{ }\mu\text{M}$; Figure 1). None of the inhibitors caused an increase in either the resting tone of the arteries or the response to the pre-contracting concentration of NA.

Incubation with indomethacin (Figure 1a) did not alter the magnitude (E_{max} , $97.56 \pm 1.83\%$, $n=6$) or sensitivity ($-\log\text{IC}_{50}$, 7.24 ± 0.20 , $n=6$) of ACh-evoked relaxation when compared with controls ($90.80 \pm 4.69\%$, $P=0.18$ and 7.23 ± 0.25 , $P=0.96$, respectively; $n=6$). In contrast, exposure to L-NOARG (Figure 1b) resulted in a significant ($P < 0.0001$), although not total, reduction in maximum relaxation ($61.68 \pm 3.38\%$, $n=6$) compared with controls ($91.55 \pm 3.95\%$, $n=6$) with a corresponding reduction in sensitivity ($-\log\text{IC}_{50}$, 6.41 ± 0.10 vs 7.19 ± 0.13 , respectively, $P < 0.005$; $n=6$). Arteries exposed to the combination of L-NOARG plus ChTx and apamin demonstrated almost total attenuation of ACh-mediated relaxation (E_{max} , $15.2 \pm 10.5\%$, $n=6$) despite producing a full concentration-response curve before exposure to these inhibitors (E_{max} , $92.59 \pm 3.65\%$, $P < 0.002$; $-\log\text{IC}_{50}$, 7.70 ± 0.30 , $n=6$). These arteries maintained their ability to relax in response to exogenous NO, as SIN-1 ($100\text{ }\mu\text{M}$) caused complete relaxation in the presence of L-NOARG, ChTx and apamin ($127.6 \pm 13.0\%$; $n=3$).

Comparison of K^{+} -induced relaxation with the EDHF-mediated component of ACh-evoked relaxation

Relaxation responses were obtained using potassium in 10 arteries with an intact endothelium and responses to ACh were also tested in nine of these. Typical relaxation responses

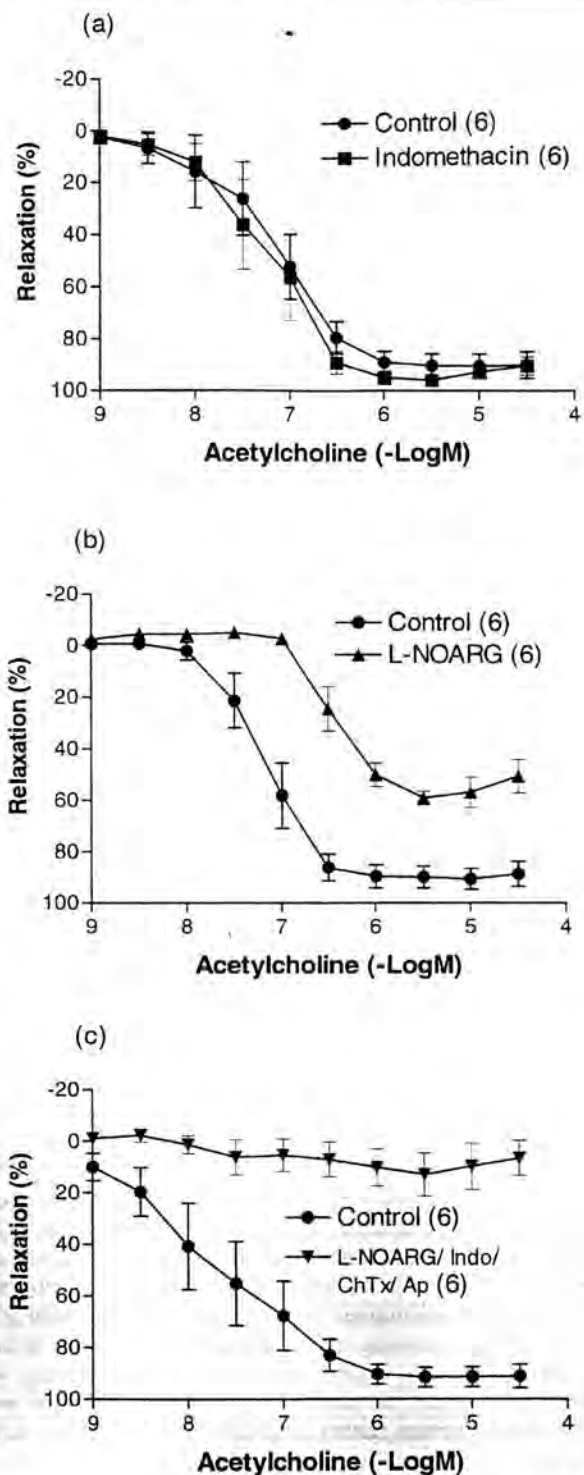


Figure 1 Cumulative concentration-response curves to ACh (10^{-9} – $3 \times 10^{-5}\text{ M}$) before and after incubation with the following combination of inhibitors (a) the cyclooxygenase inhibitor indomethacin ($10\text{ }\mu\text{M}$ for 45 min), (b) the NO synthase inhibitor L-NOARG ($100\text{ }\mu\text{M}$ for 45 min) or (c) L-NOARG ($100\text{ }\mu\text{M}$ for 45 min) plus the K^{+} channel blockers ChTx (50 nM for 10 min) and apamin (30 nM for 10 min). Results are shown as mean \pm s.e.mean, for (n) arteries.

were obtained with ACh (E_{max} , $99.07 \pm 9.61\%$; $-\log\text{IC}_{50}$, 7.03 ± 0.224 ; $n=9$), which produced a sustained concentration-dependent relaxation (Figure 2). In contrast, although

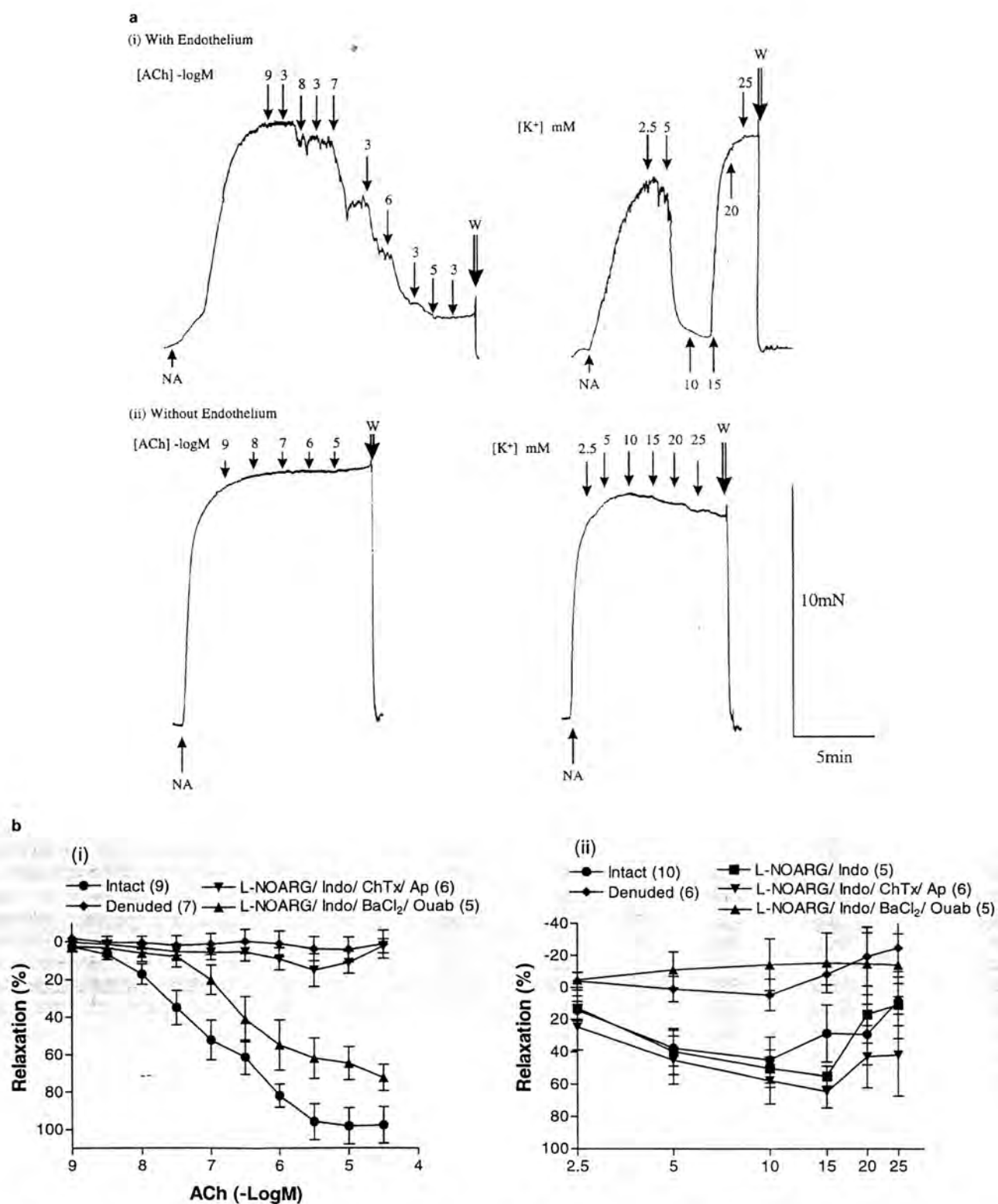


Figure 2 Comparison of ACh- and KCl-mediated relaxation. (a) Representative traces showing (i) relaxation responses of an intact artery to acetylcholine and KCl and (ii) the effect of removal of the endothelium on these responses. (b) Cumulative concentration-response curves for (i) acetylcholine and (ii) KCl obtained in arteries with and without an intact endothelium or in the presence of L-NOARG (100 μ M) and indomethacin (10 μ M) alone or combined with either ChTx (50 nM) and apamin (30 nM) or BaCl₂ (30 μ M) plus ouabain (1 mM). Results are shown as mean \pm s.e.mean, for (*n*) arteries.

potassium also relaxed these resistance arteries (E_{\max} , $74.14 \pm 5.61\%$, IC_{50} , 6.09 ± 1.17 mM; $-\log IC_{50}$, 2.12 ± 0.07 , $n=10$), the response to this compound (Figure 2) was inconsistent and was superseded by a reversal of the initial relaxation response as the concentration of KCl rose ($>15-25$ mM). As expected, removal of the endothelium virtually abolished responses to ACh ($13.34 \pm 6.16\%$, $n=7$, $P<0.0001$) but also abolished potassium-mediated relaxation (E_{\max} , $15.53 \pm 9.18\%$, $n=6$, $P<0.001$) (Figure 2).

The potassium-induced relaxation was not affected by incubation with L-NOARG and indomethacin (E_{\max} , $68.1 \pm 5.6\%$, $P=0.51$; IC_{50} , 5.74 ± 1.86 mM; $-\log IC_{50}$, 2.34 ± 0.15 , $P=0.33$, $n=5$) or with the combination of L-NOARG with indomethacin, ChTx and apamin (E_{\max} , $86.61 \pm 14.02\%$; $P=0.35$; IC_{50} , 6.78 ± 2.90 mM; $-\log IC_{50}$, 2.68 ± 0.52 , $P=0.23$, $n=6$). Indeed the maximum relaxation evoked by potassium tended to be larger in the latter group. Exposure of vessels to the combination of BaCl₂ and ouabain resulted in an increase in basal tone of 0.40 ± 0.17 mN (equivalent to $16.6 \pm 7.4\%$ of the maximum response to KPSS; $n=11$). This tended to be larger in arteries used for producing responses to ACh ($22.4 \pm 17.6\%$ KPSS; $n=5$) than in those subsequently exposed to KCl ($12.0 \pm 5.6\%$ KPSS; $n=6$). Once this contraction had stabilized, vessels were contracted with sufficient NA ($0.1-3$ μ M) to produce a contraction 60–80% the size of the maximum response to KPSS (responses to ACh obtained in one artery were discarded as the combination of BaCl₂ plus ouabain produced a contraction equivalent to 80% of the response to KPSS). Potassium-induced relaxation was totally abolished by incubation with the combination of L-NOARG with indomethacin, BaCl₂ and ouabain ($5.7 \pm 2.6\%$; $n=6$; $P<0.0001$). In contrast, a considerable ACh-induced relaxation remained evident following exposure to this combination of inhibitors although there was a trend towards reduced relaxation that did not achieve significance (E_{\max} , $76.09 \pm 8.92\%$; $P=0.16$; $-\log IC_{50}$, 6.47 ± 0.23 ; $P=0.11$, $n=5$).

Discussion

Previous investigations have demonstrated that an NO/PG-independent component of ACh-evoked relaxation is mediated by EDHF (Nakashima *et al.*, 1993; Urakami-Harasawa *et al.*, 1997; Wallerstedt & Bodelsson, 1997). Studies in arteries from experimental animals have suggested that K⁺ accounts for EDHF activity (Edwards *et al.*, 1998). In order to clarify whether K⁺ acts as an EDHF in human arteries, this investigation compared potassium-induced and EDHF-induced relaxation responses in subcutaneous resistance arteries isolated from biopsies of gluteal fat. The characteristics of potassium-induced relaxation were different from the EDHF-mediated response and, of significance, were abolished by removal of the endothelium. Taken together, this suggests that release of endothelium-derived K⁺ into the myoendothelial space does not account for EDHF activity in human subcutaneous resistance arteries.

Comparison with previous investigations indicates that the ChTx/ apamin-sensitive, NO-independent component of ACh-evoked relaxation is mediated by EDHF. In rat mesenteric arteries contracted with an α -adrenoceptor

agonist, the NO-independent component of ACh-mediated relaxation was caused by smooth muscle cell hyperpolarisation (Plane & Garland, 1996). This response is abolished by the combination of ChTx and apamin (Zygmunt & Högestätt, 1996), probably by inhibition of BK_{Ca} and SK_{Ca} on the endothelium (Doughty *et al.*, 1999). The persistence of a significant NO-independent (EDHF-mediated) relaxation in response to ACh is consistent with previous studies of human subcutaneous (Woolfson & Poston, 1990; Deng *et al.*, 1995; Hillier *et al.*, 1998), omental (Ohlmann *et al.*, 1997), gastroepiploic (Urakami-Harasawa *et al.*, 1997), coronary (Nakashima *et al.*, 1993) and pial (Pettersson *et al.*, 1995) arteries. Incomplete inhibition is unlikely to account for residual relaxation as a lower concentration of L-NOARG (3×10^{-5} M) abolished ACh-induced, endothelium-dependent relaxation in the rat aorta, pulmonary and iliac arteries (Nagao *et al.*, 1992). Furthermore, incomplete inhibition of ACh-mediated relaxation was not overcome by increasing the concentration of L-NOARG (100–300 μ M; Brandes *et al.*, 1997) or by the combined application of two different L-arginine analogues (Plane & Garland, 1996; Plane *et al.*, 1997). The failure of indomethacin to attenuate ACh-mediated relaxation in the present study confirms that prostanoids do not contribute to this response in the human gluteal, subcutaneous resistance artery. This is also consistent with previous studies, in our own and other laboratories, in which indomethacin was shown to have no effect on ACh- or bradykinin-mediated relaxation of human gluteal resistance arteries when applied alone or in combination with NO synthase inhibitors (Hillier *et al.*, 1998; Buckley *et al.*, 1999). The mechanism of endothelium-dependent relaxation of human resistance arteries may depend upon the origin of a particular vessel, however, as bradykinin-mediated relaxation of human omental arteries has an indomethacin-sensitive component which becomes evident in the presence of an NO inhibitor (Ohlmann *et al.*, 1997).

The ability of exogenous potassium to relax human gluteal resistance arteries compares with results obtained in resistance arteries from experimental animals (Edwards *et al.*, 1998; Quignard *et al.*, 1999; Doughty *et al.*, 2000; Lacy *et al.*, 2000). The identification of K⁺ as an EDHF in the earlier study was based on a comparison with the NO/PG-independent component of the response to ACh (Edwards *et al.*, 1998); responses to both ACh and exogenous K⁺ were abolished by inhibition of K_{IR} and Na⁺/K⁺ ATPase, indicating a common mechanism. Exogenous K⁺, however, produced an endothelium-independent hyperpolarization of smooth muscle cells that was unaffected by the combination of ChTx and apamin. This is consistent with ACh stimulating release of K⁺ from endothelial cells *via* ChTx/ apamin-sensitive channels. In the present study, however, the characteristics of potassium-induced and EDHF-mediated relaxation were different: whereas the ACh-induced relaxation was highly reproducible and sustained, relaxation responses to potassium were more variable and reversed readily at higher K⁺ concentrations. This is consistent with a recent study showing that exogenous K⁺ will only produce a reproducible, sustained relaxation of rat resistance arteries if they are bathed in a Krebs's solution lacking K⁺ ions (Lacy *et al.*, 2000). More striking, however, was the demonstration that, as in the rat mesenteric (Lacy *et al.*, 2000) and renal (Jiang & Dusting, 2001) arteries, potassium-mediated relaxa-

tion of human subcutaneous arteries was abolished by removal of the endothelium. This indicates an obligatory role for the endothelium in K^+ -mediated relaxation, suggesting that it may be mediated by a further endothelium-derived factor or is dependent upon myoendothelial gap junctions (Doughty *et al.*, 2000). Finally, the inability of barium and ouabain to inhibit ACh-mediated relaxation, whilst abolishing responses to potassium, indicated that these compounds caused relaxation via different mechanisms. This observation contrasts with the study by Edwards *et al.* (1998) but is consistent with data obtained in subsequent investigations (Quignard *et al.*, 1999; Lacy *et al.*, 2000). The ability of K^+ to relax only a proportion (~30%) of rat mesenteric resistance arteries in one study (Doughty *et al.*, 2000) suggests that this response may even vary in different regions

of the same artery, possibly reflecting variations in K_{IR} and Na^+/K^+ -ATPase activity (Albarwani *et al.*, 1995).

In conclusion, this investigation demonstrated an NO/PG-independent response to ACh in human subcutaneous arteries which had characteristics consistent with EDHF-mediated relaxation. The failure of exogenous potassium to produce an endothelium-independent relaxation which mimicked this response indicates that potassium is not an EDHF in these vessels.

This work was supported by a project grant from The British Diabetic Association (Diabetes, U.K.). P. Hadoke is a BHF-funded Research Fellow and B.R. Walker is a British Heart Foundation Senior Research Fellow.

References

- AALKJAER, C., HEAGERTY, A.M., PETERSEN, K.K., SWALES, J.D. & MULVANY, M.J. (1987). Evidence for increased media thickness, increased neuronal amine uptake and depressed excitation-contraction coupling in isolated resistance vessels from essential hypertensives. *Circ. Res.*, **61**, 181–186.
- ALBARWANI, S., HEINERT, G., TURNER, J.L. & KOZLOWSKI, R.Z. (1995). Differential K^+ channel distribution in smooth muscle cells isolated from the pulmonary artery tree of the rat. *Biochem. Biophys. Res. Commun.*, **208**, 183–189.
- BRANDES, R.P., BEHRA, A., LEBHERZ, C., BÖGER, R.H., BODE-BÖGER, S.M., PHIVTHONG-HGAM, L. & MÜGGE, A. (1997). NG-nitro-L-arginine- and indomethacin-resistant endothelium-dependent relaxation in the rabbit renal artery: effect of hypercholesterolemia. *Atherosclerosis*, **135**, 49–55.
- BRAYDEN, J.E. (1990). Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilation. *Am. J. Physiol.*, **259**, H668–H673.
- BUCKLEY, C., HADOKÉ, P.W.F., HENRY, E. & O'BRIEN, C.J. (1999). Altered balance of endothelium-derived relaxing factors in the acetylcholine-mediated relaxation of subcutaneous arteries isolated from patients with normal pressure glaucoma. *Br. J. Pharmacol.*, **128**, (Proc Suppl.), P264.
- CHAYTOR, A.T., EVANS, W.H. & GRIFFITH, T.M. (1998). Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. *J. Physiol.*, **508**, 561–573.
- DENG, L.-Y., LI, J.-S. & SCHIFFRIN, L. (1995). Endothelium-dependent relaxation of small arteries from essential hypertensive patients: mechanisms and comparison with normotensive subjects and with responses of vessels from spontaneously hypertensive rats. *Clin. Sci.*, **88**, 611–622.
- DOUGHTY, J.M., BOYLE, J.P. & LANGTON, P. (2000). Potassium does not mimic EDHF in rat mesenteric arteries. *Br. J. Pharmacol.*, **130**, 1174–1182.
- DOUGHTY, J.M., PLANE, F. & LANGTON, P.D. (1999). Charybdotoxin and apamin block EDHF in rat mesenteric artery if selectively applied to the endothelium. *Am. J. Physiol.*, **276**, H1107–H1112.
- EDWARDS, G., DORA, K.A., GARDENER, M.J., GARLAND, C.J. & WESTON, A.H. (1998). K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature*, **396**, 269–272.
- FELETOU, M. & VANHOUTTE, P.M. (1997). EDHF exists. *Br. J. Pharmacol.*, **122**, (Suppl.), U142.
- FURCHGOTT, R.F. & VANHOUTTE, P.M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB J.*, **3**, 2007–2018.
- HECKER, M., BARA, A.T., BAUERSACHS, J. & BUSSE, R. (1994). Characterisation of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *J. Physiol.*, **481**, 407–414.
- HILLIER, C., MCGEE, A. & McGRATH, J.C. (1998). Bradykinin-induced vasorelaxation in both diabetic and non-diabetic human resistance arteries does not involve prostacyclin. *J. Vasc. Res.*, **35**, 6–6 (Suppl.1), [Abstract].
- JIANG, F. & DUSTING, G.J. (2001). Endothelium-dependent vasorelaxation independent of nitric oxide and K^+ release in isolated renal arteries of rats. *Br. J. Pharmacol.*, **132**, 1558–1564.
- LACY, P.S., PILKINGTON, G., HANVESAKUL, R., FISH, H.J., BOYLE, J.P. & THURSTON, H. (2000). Evidence against potassium as an endothelium-derived hyperpolarizing factor in rat mesenteric small arteries. *Br. J. Pharmacol.*, **129**, 605–611.
- MATOKA, T., SHIMOKAWA, H., NAKASHIMA, M., HIRAKAWA, Y., MUKAI, Y., HIRANO, K., KANAIDE, H. & TAKESHITA, A. (2000). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J. Clin. Invest.*, **106**, 1521–1530.
- MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.*, **41**, 19–26.
- NAGAO, T., ILLIANO, S. & VANHOUTTE, P.M. (1992). Heterogeneous distribution of endothelium-dependent relaxations resistant to N^G -nitro-L-arginine in rats. *Am. J. Physiol.*, **263**, H1090–H1094.
- NAKASHIMA, M., MOMBOULI, J.-V., TAYLOR, A.A. & VANHOUTTE, P.M. (1993). Endothelium-dependent hyperpolarization caused by bradykinin in human coronary arteries. *J. Clin. Invest.*, **92**, 2867–2871.
- OHLMANN, P., MARTINEZ, M.C., SCHNEIDER, F., STOCLET, J.C. & ANDRIANTSITOHAINA, R. (1997). Characterization of endothelium-derived relaxing factors released by bradykinin in human resistance arteries. *Br. J. Pharmacol.*, **121**, 657–664.
- PETERSSON, J., ZYGMUNT, P.M., BRANDT, L. & HOGESTATT, E.D. (1995). Substance P-induced relaxation and hyperpolarization in human cerebral arteries. *Br. J. Pharmacol.*, **115**, 889–894.
- PLANE, F. & GARLAND, C.J. (1996). Influence of contractile agonists on the mechanism of endothelium-dependent relaxation in rat isolated mesenteric artery. *Br. J. Pharmacol.*, **119**, 191–193.
- PLANE, F., HOLLAND, M., WALDRON, C.J., GARLAND, C.J. & BOYLE, J.P. (1997). Evidence that anandamide and EDHF act via different mechanisms in rat isolated mesenteric arteries. *Br. J. Pharmacol.*, **121**, 1509–1511.
- QUIGNARD, J.-F., FELETOU, M., THOLON, C., VILANE, J.-P., DUHAULT, J. & VANHOUTTE, P.M. (1999). Potassium ions and endothelium-derived hyperpolarizing factor in guinea-pig carotid and porcine coronary arteries. *Br. J. Pharmacol.*, **127**, 27–34.
- RANDALL, M.D., ALEXANDER, S.P.H., BENNET, T., BOYD, E.A., FRY, J.R., GARDINER, S.M., KEMP, P.A., MCCULLOCH, A. & KENDAL, D.A. (1996). An exogenous cannabinoid as an endothelium-derived vasorelaxant. *Biochem. Biophys. Res. Commun.*, **229**, 114–120.
- SHIMOKAWA, H., YASUTAKE, H., FUJII, K., OWADA, M.K., NAKAIKE, R., FUKUMOTO, Y., TAKAYANAGI, T., NAGAO, T., EGASHIRA, K., FUJISHIMA, M. & TAKESHITA, A. (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J. Cardiovasc. Pharmacol.*, **28**, 703–711.
- TAYLOR, S.G. & WESTON, A.H. (1988). Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol. Sci.*, **9**, 272–274.

- URAKAMI-HARASAWA, L., SHIMOKAWA, H., NAKASHIMA, M., EGASHIRA, K. & TAKESHITA, A. (1997). Importance of endothelium-derived hyperpolarizing factor in human arteries. *J. Clin. Invest.*, **100**, 2793–2799.
- WALLERSTEDT, S.M. & BODELSSON, M. (1997). Endothelium-dependent relaxation by substance P in human isolated omental arteries and veins: relative contribution of prostanoids, nitric oxide and hyperpolarization. *Br. J. Pharmacol.*, **120**, 25–30.
- WOOLFSON, R.G. & POSTON, L. (1990). Effect of N^G-monomethyl-L-arginine on endothelium-dependent relaxation of human subcutaneous resistance arteries. *Clin. Sci.*, **79**, 273–278.
- ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1996). Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. *Br. J. Pharmacol.*, **117**, 1600–1606.

(Received October 12, 2000

Revised April 24, 2001

Accepted April 30, 2001)

Preservation of vascular function in rat mesenteric resistance arteries following cold storage, studied by small vessel myography

C.A. McIntyre, B.C. Williams, ¹R.M. Lindsay, ¹J.A. McKnight & ^{2,3}P.W.F. Hadoke

Department of Medicine and ¹Metabolic Unit, Western General Hospital, and ²Department of Medicine, Royal Infirmary, Edinburgh, Scotland

1 The use of isolated blood vessels to investigate the physiological and pharmacological control of the vasculature is limited by the requirement to use freshly isolated vessels. Hence, the aim of this study was to determine whether vascular smooth muscle and endothelial cell function could be preserved in resistance arteries by storing them in physiological salt solution (PSS) at 4°C.

2 Third order mesenteric resistance arteries (mean internal diameter $237 \pm 6 \mu\text{m}$) were dissected from the mesenteric bed of male Cob-Wistar rats. The vessel segments were mounted in a small vessel myograph for measurement of isometric tension, and equilibrated at their optimum resting force. Contractile responses to noradrenaline (NA; 1×10^{-9} – 3×10^{-5} M), phenylephrine (PE, 1×10^{-9} – 3×10^{-5} M), potassium chloride (KCl; 2.5–140 mM) and endothelin (ET-1, 1×10^{-11} – 3×10^{-7} M) and relaxant responses to acetylcholine (ACh; 1×10^{-9} – 3×10^{-5} M) and 3-morpholinolysynonimine (SIN-1; 1×10^{-9} – 1×10^{-4} M) were obtained in arteries, immediately after dissection (day 0) and following one to four days storage (day 1–day 4).

3 All arteries produced concentration-dependent contractions in response to each of the vasoconstrictors. There were no significant differences in the magnitude or sensitivity (pD_2) of the vasoconstrictor responses between fresh and stored vessels.

4 Arteries precontracted with NA to approximately 80% of the maximum response, relaxed in a concentration-dependent manner in response to ACh and SIN-1. Vessel storage for up to three days resulted in no change in response to ACh or SIN-1.

5 Vessels analysed after four days of storage demonstrated a significant increase in sensitivity to ACh and SIN-1 ($-\log\text{IC}_{50}$ (M) values; ACh; day 0, 7.46 ± 0.13 vs day 4, 7.97 ± 0.11 , $P < 0.01$ and SIN-1; day 0, 4.87 ± 0.10 vs day 4, 5.52 ± 0.08 , $P < 0.01$). There was also a significant increase in the maximum relaxant response to ACh after four days of storage (% relaxation; day 0, 92.65 ± 2.84 vs day 4, 100.36 ± 0.36 , $P < 0.05$).

6 These results demonstrate that small resistance arteries remain viable if stored in PSS at 4°C for up to four days, with no loss in endothelial cell function. The altered sensitivity to the vasodilators on day 4 suggests that vessels should only be stored for up to three days following dissection for analysis of functional responses.

Keywords: Mesenteric resistance arteries; endothelium; vascular smooth muscle; cold storage

Introduction

Isolated blood vessel segments are used extensively to investigate *in vitro* the physiological and pharmacological control of the vasculature and the mechanisms responsible for deranged vascular function in disease states. One of the major limitations of this type of investigation is the perceived need to use freshly isolated blood vessels. This is mainly due to the well-documented fragility of the vascular endothelium, which may be damaged both during isolation and storage (Kristek *et al.*, 1993; Torok *et al.*, 1993), in contrast to smooth muscle which can remain viable for up to eight days when stored at 2–6°C (Kristek *et al.*, 1993). The requirement that blood vessels must be freshly isolated restricts the number of investigations which can be performed on vessel segments from a single animal and is even more problematic when human blood vessels are studied, since the availability of such vessels can be both irregular and unpredictable. Consequently, a straightforward and reliable method for storing vessels before experimentation is desirable and this idea has stimulated investigations into both refrigeration (2–6°C; Shibata, 1969; Carrier *et al.*, 1973; Kristek *et al.*, 1993; Torok *et al.*, 1993) and

low temperature (–70 or –190°C) cryopreservation of blood vessel samples (Muller-Schwienitzer *et al.*, 1986; Ku *et al.*, 1990).

Most studies carried out to date on the effects of storage of blood vessels on vascular reactivity have been performed with large conduit vessels (Shibata, 1969; Carrier 1973; Kristek *et al.*, 1993; Torok *et al.*, 1993) rather than small vessels. However, following the introduction of small vessel myography (Mulvany & Halpern, 1977), it has become desirable to preserve vascular function in small resistance arteries for a prolonged period of time. The aim of the experiments described in this study was, therefore, to determine whether vascular smooth muscle and endothelial cell function could be preserved in resistance arteries by storing them in physiological salt solution (PSS) at 4°C.

Methods

Experimental set-up

Male Cob-Wistar rats (150–250 g) were killed by asphyxiation with CO₂ followed by cervical dislocation. The mesenteric bed was removed and placed in cold (4°C) PSS of the following

³ Author for correspondence at: Department of Medicine (RIE), The University of Edinburgh, Royal Infirmary, Lauriston Place, Edinburgh EH3 9YW.

composition (mM): NaCl 119, KCl 4.7, CaCl_2 2.5, MgSO_4 1.17, NaHCO_3 25, KH_2PO_4 1.18, ethylenediaminetetra-acetic acid di-potassium salt (K_2EDTA) 0.026 and D-glucose 5.5. Third order branches of the mesenteric artery (mean internal diameter; $237 \pm 6 \mu\text{m}$, 107 vessels from 32 rats) were dissected from the mesenteric bed and cleaned of connective tissues under a light microscope. One was used immediately after dissection (day 0) whilst the others were stored separately (at 4°C , in vials containing 1 ml PSS) for up to four days (day 1–day 4).

The mesenteric arterial vessel segments were mounted in a small vessel, dual chamber, myograph (chamber volume 12.5 ml) for measurement of isometric tension. Two myographs were used in parallel, allowing investigation of four vessels each day. Each vessel segment, approximately 2 mm in length, was mounted on two $40 \mu\text{m}$ stainless steel wires, one of which was attached to a force transducer and the other to a micrometer. The length of each vessel segment was measured by a light microscope, with the vessel segment bathed in PSS at 37°C , bubbled with 95% O_2 and 5% CO_2 .

Following equilibration for 30 min, stepwise radial stretching was performed to determine the lumen diameter necessary for optimal force generation. This was achieved by applying the LaPlace relationship as described by Mulvany & Halpern (1977). The vessel segment was then stretched to achieve 90% of the diameter expected if it had been relaxed and exposed to a transmural pressure of 13.3 kPa (100 mmHg). This resting tension has been previously shown to produce maximal force generation in the rat mesenteric artery (Buus *et al.*, 1994; Falloon *et al.*, 1995).

The vessel segment was allowed to equilibrate for a further 30 min before the viability was assessed by use of a standard start procedure. This consisted of stimulating twice with KPSS (125 mM, made by equimolar substitution of KCl for NaCl in PSS) containing 10^{-5} M noradrenaline (NA), then once with KPSS alone and once with 10^{-5} M NA alone. Finally the vessel segment was stimulated for a third time with NA-KPSS. The vessel segment was activated for 2 min with each solution followed by a 5 min washout period in PSS to allow full relaxation. The measurement of viability is performed in these vessels to determine whether the isolation and mounting of the vessel damaged the arterial wall. This is achieved by calculating the effective pressure induced by the contractile agonist from the LaPlace relation (effective pressure = wall tension/(internal circumference/ 2π)), which corrects the small differences in the length and diameter of the vessel segments (Mulvany & Halpern, 1977). Exposure of rat mesenteric arteries usually produces a response >20 kPa and, by convention, arteries are considered unviable (damaged) if the effective pressure is less than 13.3 kPa.

Protocols

The ability of the vessel to respond to contractile and dilator agents was investigated by producing cumulative concentration-response curves (CCRCs). CCRCs were obtained for noradrenaline (NA; 1×10^{-9} – 3×10^{-5} M), acetylcholine (ACh; 1×10^{-9} – 3×10^{-5} M), 3-morpholinysydnonimine (SIN-1; 1×10^{-9} – 1×10^{-4} M), phenylephrine (PE; 1×10^{-9} – 3×10^{-5} M), potassium chloride (KCl; 2.5–140 mM) and endothelin-1 (ET-1; 1×10^{-11} – 3×10^{-7} M). After each CCRC the vessel was washed four times with PSS and allowed a 15 min equilibration period in PSS before exposure to the next agent. Responses to vasodilator agents were determined following production of a stable contraction with $3 \mu\text{M}$ NA (which induced approximately 80% maximum contraction).

Drugs

All salts were obtained from BDH Laboratory Supplies (Poole, Dorset). Acetylcholine chloride, noradrenaline bitartrate, phenylephrine hydrochloride were from Sigma (Poole, Dorset). SIN-1 was a gift from Dr K. Schonafinger (Cassella, Germany) and endothelin-1 was obtained from Calbiochem-Novabiochem (U.K.) Ltd, (Beeston, Nottingham).

All drugs were dissolved in distilled water with the exception of ET-1. Endothelin stock was dissolved in 5% ethanol. Further dilutions were made in 50% methanol/50% distilled water, divided into aliquots and stored as 10^{-5} M stock solutions at -20°C . On the day of use a stock solution was thawed and subsequent dilutions were made in distilled water. These solutions were added directly to the PSS in the myograph chamber to give the final dilutions.

Statistics

All values presented are mean \pm s.e. mean for n experiments. NA, PE, KCl and ET-1-induced tension is expressed as a percentage of the maximum contractile response to that agent. The relaxation to ACh and SIN-1 is expressed as a percentage of the NA-induced precontraction.

Sensitivity to the agent is expressed as the negative log of the effective concentration (M) of the drug required to produce 50% of the maximum effect (pD_2 for vasoconstrictors and $-\log \text{IC}_{50}$ for vasodilators). The sensitivity was calculated from each concentration-response curve by fitting the Hill equation by use of a curve fitting programme (Fig.P; Biosoft, Cambridge, U.K.).

Student's independent samples t test was employed in the statistical comparison between the sensitivity and maximum responses (relaxation or contraction) of CCRCs in fresh and stored vessels. Significance was assumed if $P < 0.05$.

Results

The standard start procedure demonstrated that the vessels analysed immediately following dissection were viable and that this viability was unaffected by storage over a four day period (Table 1). Indeed, all the arteries used in this investigation were shown to be viable. The arteries produced strong, concentration-dependent, contractions in response to each of the vasoconstrictors, NA, PE, KCl and ET-1. The magnitude and sensitivity of these responses were unaffected by storage (Table 2a).

The concentration of noradrenaline required to produce approximately 80% of the maximum contractile response in freshly isolated vessels was $3 \mu\text{M}$. This concentration was used to precontract the tissues in order to assess the relaxant properties of the vasodilator agents. The contraction produced by this concentration of NA was unchanged by storage for two days but was significantly reduced on day 3 (Table 3). However, the reduction observed on day 3 was not sustained on day 4.

Following precontraction with $3 \mu\text{M}$ NA, ACh and SIN-1 both induced concentration-dependent relaxations which were still evident after four days of storage. Storage of the vessels for up to three days resulted in no change in the responses induced by these agents (Table 2b). The magnitude and sensitivity of the relaxations was unchanged until the fourth day of storage, when the arteries demonstrated a significant increase in the maximum response to ACh ($P < 0.05$) and a significant increase in sensitivity ($P < 0.01$) to both ACh and

Table 1 The effect of prolonged cold storage on the viability of rat mesenteric resistance arteries

	Viability (kPa)
Day 0	26.33 ± 1.78 (24)
Day 1	29.14 ± 2.56 (22)
Day 2	27.87 ± 2.08 (24)
Day 3	29.66 ± 2.76 (20)
Day 4	31.42 ± 2.55 (17)

Values are mean ± s.e. mean, *n* numbers in parentheses. The viability value is an indication of the effective pressure developed by the artery in response to a solution containing high concentrations of potassium (125 mM) and NA (10^{-5} M), which produces a maximal contraction. It is calculated from the LaPlace relation (effective pressure = wall tension / (internal circumference / (2π))) and is used routinely to determine whether a vessel has been damaged during isolation. In the present investigation measurement of viability also provided an indication of whether prolonged storage had caused a loss of viability. Rat mesenteric arteries usually produce an effective pressure >20 kPa and are considered unviable if the effective active pressure developed in response to NA-KPSS is less than 13.3 kPa (100 mmHg). All the vessels mounted in the organ bath for the present study were shown to be viable.

SIN-1 (Table 2b). A trend towards an increased maximum response to SIN-1 was also observed, but this was not significant.

Discussion

The results of this study indicate that rat mesenteric resistance arteries remain viable if stored in PSS at 4°C for up to four days. The sensitivity of the arteries to the vasodilators, ACh and SIN-1, remained stable following the first three days of cold storage and thereafter increased on day 4, whereas responses to vasoconstrictor agonists were unaltered.

Vasodilator function

Previous investigations have shown that cells in the vascular wall undergo time-dependent alterations when stored at 4°C. The severity and speed of these changes vary between different cell types with the earliest alterations occurring in nerve fibres and endothelial cells (Kristek *et al.*, 1993). These morphological alterations are accompanied by functional abnormalities, with endothelium-dependent relaxation reduced after as little as 24 h storage (Flanders *et al.*, 1996). Similarly, cryopreservation, which involves storing the tissues in dimethylsulphoxide (DMSO) at either -70 or -190°C, produces endothelial cell dysfunction (Ku *et al.*, 1990). In addition to the problems this poses for pharmacological investigation, such endothelial cell dysfunction is a cause for concern for bypass grafting and organ transplantation. Consequently the preservative effects of a variety of solutions have been investigated. Whilst this has produced evidence that some preservation solutions (University of Wisconsin, St Thomas' Hospital) are better for preserving endothelial function in rabbit aorta (Eberl *et al.*, 1993) and porcine hepatic artery (Flanders *et al.*, 1996), the reasons for these differences are unclear. Indications into the protective action of these solutions are not provided by comparison of their contents. University of Wisconsin (Flanders *et al.*, 1996) solution is a calcium-free, high potassium solution which contains agents which inhibit free radical generation (adenosine, allopurinol, glutathione) and platelet aggregation (adenosine). In contrast, St. Thomas'

Table 2 The effects of cold storage on the maximum contraction (a) or relaxation (b) of rat 3rd order mesenteric arteries to vasoconstrictors (a) or vasodilators (b)

	Maximum response (mN mm ⁻¹)					Maximum response (% relaxation)				
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 0	Day 1	Day 2	Day 3	Day 4
a	NA	2.79 ± 0.28	2.52 ± 0.31	3.06 ± 0.32	2.26 ± 0.18	2.09 ± 0.28	5.76 ± 0.08	5.85 ± 0.05	5.89 ± 0.09	5.97 ± 0.10
	PE	2.91 ± 0.16	3.39 ± 0.25	3.59 ± 0.41	2.73 ± 0.34	2.76 ± 0.45	5.60 ± 0.09	5.60 ± 0.10	5.47 ± 0.06	5.74 ± 0.17
	KCl	1.35 ± 0.07	1.44 ± 0.18	1.79 ± 0.31	1.50 ± 0.21	1.83 ± 0.27	1.58 ± 0.04	1.62 ± 0.04	1.61 ± 0.04	1.54 ± 0.04
	ET-1	2.90 ± 0.33	3.23 ± 0.25	2.64 ± 0.42	2.91 ± 0.35	2.57 ± 0.29	8.45 ± 0.18	8.50 ± 0.09	8.66 ± 0.11	8.39 ± 0.13
b	ACh	92.65 ± 2.84	94.28 ± 1.96	96.06 ± 2.79	90.97 ± 4.82	100.36 ± 0.36*	7.46 ± 0.13	7.27 ± 0.16	7.41 ± 0.12	7.97 ± 0.11†
	SIN-1	84.84 ± 6.56	90.72 ± 3.48	94.33 ± 2.82	96.21 ± 1.62	96.12 ± 2.08	4.87 ± 0.10	4.88 ± 0.10	5.07 ± 0.10	5.52 ± 0.08‡

Values are mean ± s.e. mean, *n* = 10. **P* < 0.05 when compared with maximum response for day 0 (ACh) and *P* < 0.01 when compared with -logIC₅₀ value for day 0 (†ACh & ‡SIN-1), by use of Student's independent samples *t* test.

Table 3 The effect of cold storage on the magnitude of noradrenaline ($3 \mu\text{M}$)-induced precontraction in rat mesenteric resistance artery

	Precontraction (mN mm^{-1})	
	ACh	SIN-1
Day 0	1.91 ± 0.15	$2.24 \pm 0.19^*$
Day 1	2.47 ± 0.23	2.47 ± 0.17
Day 2	2.07 ± 0.20	2.31 ± 0.16
Day 3	$1.45 \pm 0.15^*$	$1.63 \pm 0.21^\dagger$
Day 4	1.92 ± 0.42	1.72 ± 0.45

Values are mean \pm s.e. mean, $n=10$. $P < 0.05$ when compared with precontraction value for day 0. *ACh and † SIN-1, by use of Student's independent samples t test.

solution is a simple salt solution containing moderately high concentrations of potassium and magnesium but not glucose (Eberl *et al.*, 1993).

In contrast to previous investigations with large conduit arteries, our results demonstrated that the rat mesenteric resistance artery retains endothelium-dependent vasodilator function throughout the period of preservation. This suggests that the endothelium in these vessels does not become damaged as rapidly as the endothelium of rabbit aortae. Indeed, the sensitivity to both endothelium-dependent and -independent dilators was increased significantly after storage for four days. The vasodilators used in this investigation both act by the production of nitric oxide (Furchgott *et al.*, 1987; Feelisch & Noack, 1987); ACh induces vasodilatation by stimulation of constitutive nitric oxide synthase (eNOS; Fostermann *et al.*, 1991) in the endothelium, whilst SIN-1 (the active metabolite of molsidomine) spontaneously releases NO by a free radical process following base-catalyzed hydrolysis to produce endothelium-independent relaxation (Feelisch, 1991). NO production causes vasodilatation by stimulation of soluble guanylate cyclase with the subsequent elevation of cyclic guanosine 3':5' monophosphate (cyclic GMP; Waldman & Murad, 1987), which lowers intracellular Ca^{2+} concentrations in the smooth muscle cells. The observed increase in sensitivity on day 4 to both ACh and SIN-1 in our studies, with no accompanying change in sensitivity to vasoconstrictors, suggests that the enzyme guanylate cyclase could have become upregulated (Moncada *et al.*, 1991).

It has been demonstrated that the basal release of NO from the endothelium *in vivo* acts to reduce the sensitivity of the vascular wall to agonist-induced dilatation (Busse *et al.*, 1989). This basal release is stimulated by shear stress activation of eNOS (Nishida *et al.*, 1992) and, therefore, is unlikely to occur in the stored arteries. Consequently, it is possible that a reduced basal release of endothelium-derived NO results in an upregulation of guanylate cyclase. However, it should be noted that endothelial cell dysfunction in stored rabbit aorta was not accompanied by an increased sensitivity to sodium nitropruside (Torok *et al.*, 1993). Alternatively, the increased sensitivity we observed to ACh and SIN-1 could be due to an increase in permeability of the vascular smooth muscle cells to nitric oxide. Previous investigations have suggested that increased smooth muscle cell permeability may develop (Torok *et al.*, 1993) and this may allow more efficient access of NO to soluble guanylate cyclase in the vascular smooth muscle cells.

It is unclear why endothelial cell function is preserved in the rat mesenteric arteries but not in larger vessels stored at a similar temperature (Kristek *et al.*, 1993; Torok *et al.*, 1993; Flanders *et al.*, 1996). The size and origin of the vessels may be important as endothelial cell function appears to vary according to the diameter of the vessel (Haefliger *et al.*,

1993), and upon its anatomical origin (Vanhoutte & Miller, 1985). Alternatively, the different storage conditions may be significant as some preservative solutions appear to more effective than others in maintaining the function of the endothelium (Eberl *et al.*, 1993; Vohra *et al.*, 1997). Further work is required to clarify the cause of these variations.

Vasoconstrictor function

The measurement of vessel viability is usually performed to ensure that vessel preparation does not damage the vessel wall. As an experienced myographer will rarely cause such damage, the inclusion of this measurement in the present study further demonstrated that prolonged storage did not impair viability. Indeed, the effective pressure (viability) measurements were remarkably consistent throughout the study (Table 1).

It has been suggested that the changes in contractile function detected in stored vessels represent a balance between the effects of endothelial cell and smooth muscle cell dysfunction (Kristek *et al.*, 1993). Consequently, the observed increase in sensitivity of the rabbit aorta to NA after preservation for three to four days (Shibata, 1969; Carrier *et al.*, 1973; Vohra *et al.*, 1997) may be the result of early endothelial cell dysfunction, as the vascular endothelium can modulate the constrictor response to many agonists. This would be similar to the increased reactivity to NA (Dohi *et al.*, 1990) documented following removal of the endothelium in mesenteric resistance arteries from normal rats. Alternatively, Kristek *et al.* (1993) suggested that storage leads to altered calcium homeostasis, as a result of increased permeability and membrane depolarization. As the time of preservation lengthens, the increased response to NA is probably balanced by an attenuation resulting from gradual impairment of contractile function.

Our investigation demonstrated that NA and PE-induced contractions in the rat mesenteric artery were unaffected by storage. It is possible that the conservation of endothelium-dependent relaxation prevented the increased responsiveness to these agonists observed by other investigators (Shibata, 1969; Carrier *et al.*, 1973; Kristek *et al.*, 1993). Furthermore, the maintenance of these responses indicated that α -adrenoceptor-mediated vasoconstriction was not altered by storage.

Despite the maintained response to NA and PE throughout storage, the precontracting concentration of NA ($3 \times 10^{-6} \text{ M}$) produced responses that were reduced on days 3 and 4. Despite the similarity of these measurements, the reduction was only significant (when compared with fresh vessels) on day 3. It is considered necessary to use a submaximal concentration of the precontracting agent for investigation of responses to vasodilators to prevent over-estimation of the sensitivity of the tissue to these agents. However, at submaximal concentrations NA often produced a biphasic response with a slight loss of tone after the initial contraction, after which the contraction stabilized. This loss of tone appeared to be more marked on days 3 and 4, accounting for the reduced contractile response, but was highly variable. The lack of significance on day 4 was a consequence of the variability of this biphasic response.

In contrast to NA, it has previously been shown that KCl-mediated vasoconstriction is significantly reduced following preservation for four days, but this reduction could be partially attenuated if preservation was performed in a high potassium Ringer solution (Shibata, 1969). This may be due to storage-induced alterations in the smooth muscle cell polarization state, as preservation has been shown to reduce significantly intracellular potassium but increase intracellular sodium and calcium (Carrier *et al.*, 1973). Our results demonstrated that

such changes were not significant in the rat mesenteric artery, as responses to KCl were unaltered.

Contractile responses to endothelin-1 following prolonged storage of the vessels have not been investigated previously. However, various insults, including hypoxia, can lead to the release of this potent vasoconstrictor by endothelial cells (reviewed in Haynes & Webb, 1993), resulting in receptor down-regulation and reduced sensitivity to this agonist (Clozel *et al.*, 1993). Consequently, it is possible that the endothelial damage described in larger vessels following storage (Kristek *et al.*, 1993; Torok *et al.*, 1993; Flanders *et al.*, 1996), would have caused reduced sensitivity to endothelin-1. The unaltered response in the rat mesenteric artery to ET-1 in our studies suggests that prolonged storage of this tissue does not lead to significant release of ET-1 from the vascular endothelium. This supports the argument that there is no significant endothelial cell damage in these vessels after storage, which is consistent with the results of our experiments with the vasodilator ACh.

Finally, the maintenance of the responsiveness to the vasoconstrictors studied suggests that the contractile activity of the smooth muscle was not attenuated during preservation. This is consistent with previous investigations carried out in rabbit conduit arteries, in which contractile function to exogenously applied vasoconstrictors was not reduced after preservation for up to eight days (Shibata, 1969; Kristek *et al.*, 1993). Indeed, Shibata (1969) demonstrated that contractile responses to NA in those vessels were only reduced significantly after 10–12 days preservation and detectable contractions were still present after twenty days storage in Ringer solution.

In summary, no comprehensive studies have previously been published in relation to the preservation of resistance

artery function after prolonged storage in physiological media. We have clearly demonstrated that rat mesenteric artery segments maintain stable responsiveness to vasoconstrictors and the endothelium-dependent and endothelium-independent vasodilators, ACh and SIN-1, respectively, for up to four days when stored in a simple PSS medium at 4°C. The increase in sensitivity observed with both ACh (endothelium-dependent) and SIN-1 (endothelium-independent) on day four suggests an increase in smooth muscle sensitivity to NO after this storage period. Preservation of the contractile responses implies that receptor function and smooth muscle cell polarization state are not adversely affected by preservation. Consequently, rat resistance artery segments can be stored for subsequent investigation and do not develop the significant endothelial cell damage previously observed in larger arteries. This should allow more efficient use of animals tissues in the future, with stored arteries being used for the purpose of training personnel in the myography procedure, or demonstrating functional responses of isolated vessels to students. Further work will be necessary in order to determine whether vascular function in human resistance artery segments, obtained from gluteal fat biopsies, can be preserved under similar conditions of cold storage.

This work was funded by the British Diabetic Association (grant number RD95/0001098). P.W.F.H. is a Department of Medicine (University of Edinburgh) funded research fellow. We are grateful to Mr W. Adams, Department of Public Health, University of Edinburgh, for advice on statistical methods.

References

- BUSSE, R., POHL, U., MULSCH, A. & BASSENCE, F. (1989). Modulation of the vasodilator action of SIN-1 by the endothelium. *J. Cardiovasc. Pharmacol.*, **14**, S81–S85.
- BUUS, N.H., VAN BAVEL, E. & MULVANY, M.J. (1994). Differences in sensitivity of rat mesenteric small arteries to agonists when studied as ring preparations or as cannulated preparations. *Br. J. Pharmacol.*, **112**, 579–587.
- CARRIER, O., MURPHY, J.C. & TENNER, T.E. (1973). Effect of cold storage on calcium-related responses and electrolyte content of rabbit aortic strips. *Eur. J. Pharmacol.*, **24**, 225–233.
- CLOZEL, M., LOFFLER, B.M., BREU, V., HILFGER, L., MAIRE, J.P. & BUTSCHA, B. (1993). Down-regulation of endothelin receptors by autocrine production of endothelin-1. *Am. J. Physiol.*, **265**, C188–C192.
- DOHI, Y., THIEL, M.A., BUHLER, F.R. & LUSCHER, T.F. (1990). Activation of endothelial L-arginine pathway in resistance arteries. *Hypertension*, **15**, 170–179.
- EBERL, T., SCHMID, T., WODLINGER, R., HENGSTER, P., HEROLD, M., WEISS, H., OBERHUBER, G., KRAUSLER, R. & MARGREITER, R. (1993). Which organ preservation solution best protects vascular endothelium? *Transplant. Proceed.*, **25**, 3019.
- FALLOON, B.J., STEPHENS, N., TULIP, J.R. & HEAGERTY, A.M. (1995). Comparison of small artery sensitivity and morphology in pressurized and wire-mounted preparations. *Am. J. Physiol.*, **268**, H670–H678.
- FEELISCH, M. (1991). The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J. Cardiovasc. Pharmacol.*, **17**, S25–S33.
- FEELISCH, M. & NOACK, E.A. (1987). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.*, **139**, 19–30.
- FLANDERS, S., HARDY, K.J. & LEW, M.J. (1996). Effect of cold storage in University of Wisconsin solution on the responses of porcine hepatic arteries to 5-hydroxytryptamine and bradykinin *in vitro*. *Br. J. Pharmacol.*, **117**, 142–146.
- FORSTERMANN, U., SCHMIDT, H.H.H.W., POLLOCK, J.S., SHENG, H., MITCHELL, J.A., WARNER, T.D., NAKANE, M. & MURAD, F. (1991). Isoforms of nitric oxide synthase. Characterisation and purification from different cell types. *Biochem. Pharmacol.*, **42**, 1849–1857.
- FURCHGOTT, R.F., CARVALHO, M.H., KHAN, M.T. & MATSUNAGA, K. (1987). Evidence for endothelium-dependent vasodilation of resistance vessels by acetylcholine. *Blood Vessels*, **24**, 145–149.
- HAEFLIGER, I.O., FLAMMER, J. & LUSCHER, T.F. (1993). Heterogeneity of endothelium-dependent regulation in ophthalmic and ciliary arteries. *Invest. Ophthalmol. Vis. Sci.*, **34**, 1722–1730.
- HAYNES, W.G. & WEBB, D.J. (1993). Editorial review: The endothelin family of peptides: local hormones with diverse roles in health and disease. *Clin. Sci.*, **84**, 485–500.
- KRISTEK, F., TOROK, J. & SIKVLOVA, J. (1993). Morphological and functional alterations in endothelium, smooth muscle and nerve fibres in rabbit aorta after storage at 4°C. *Cryobiology*, **30**, 376–385.
- KU, D.D., WILLIS, W.L. & CAULFIELD, J.B. (1990). Retention of endothelium-dependent vasodilatory responses in canine coronary arteries following cryopreservation. *Cryobiology*, **27**, 511–520.
- MONCADA, S., REES, D.D., SCHULZ, R. & PALMER, R.M.J. (1991). Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 2166–2170.
- MULLER-SCHWIENITZER, E., TAPPARELLI, C. & VICTORZON, M. (1986). Functional studies on human veins after storage at –190°C. *Br. J. Pharmacol.*, **88**, 685–687.
- MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.*, **41**, 19–26.

- NISHIDA, K., HARRISON, D.G., NAVAS, J.P., FISHER, A.A., DOCKERY, S.P., UEMATSU, M., NEREM, R.M., ALEXANDER, R.W. & MURPHY, T.J. (1992). Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J. Clin. Invest.*, **90**, 2092–2096.
- SHIBATA, S. (1969). Effect of prolonged cold storage on the contractile response of strips of rabbit aorta to various agents. *Circ. Res.*, **24**, 179–187.
- TOROK, J., KRISTEK, F. & MOKRASOVA, M. (1993). Endothelium-dependent relaxation in rabbit aorta after cold storage. *Eur. J. Pharmacol.*, **228**, 313–319.
- VANHOUTTE, P.M. & MILLER, V.M. (1985). Heterogeneity of endothelium-dependent responses in mammalian blood vessels. *J. Cardiovasc. Pharmacol.*, **7** (Suppl. 3), S12–S23.
- VOHRA, M.M., CHEDRAWY, E.G., LI, G. & SULLIVAN, J.A. (1997). Functional assessment of rabbit thoracic aorta stored in University of Wisconsin, St Thomas' Hospital and Krebs solution. *Br. J. Pharmacol.*, **120**, 153P.
- WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.*, **39**, 163–196.

(Received November 3, 1997

Revised December 22, 1997

Accepted January 12, 1998)